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*January 14, 2005*

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**PROVISIONAL APPLICATION FOR PATENT COVER SHEET**

This is a request for filing a PROVISIONAL APPLICATION FOR PATENT under 37 CFR 1.53(c).

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Additional inventors are being named on the <u>1</u> separately numbered sheets attached hereto					
TITLE OF THE INVENTION (500 characters max)					
HUMAN CATHELICIDIN ANTIMICROBIAL PEPTIDES					
Direct all correspondence to: CORRESPONDENCE ADDRESS					
<input checked="" type="checkbox"/> Customer Number: <div style="border: 1px solid black; padding: 2px; display: inline-block;">27111</div>					
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ENCLOSED APPLICATION PARTS (check all that apply)					
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METHOD OF PAYMENT OF FILING FEES FOR THIS PROVISIONAL APPLICATION FOR PATENT					
<input checked="" type="checkbox"/> Applicant claims small entity status. See 37 CFR 1.27.					
<input checked="" type="checkbox"/> A check or money order is enclosed to cover the filing fees.					
<input checked="" type="checkbox"/> The Director is hereby authorized to charge filing fees or credit any overpayment to Deposit Account Number: <u>02-4070</u>					
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FILING FEE Amount (\$) <div style="border: 1px solid black; padding: 5px; display: inline-block;">80.00</div>					
The invention was made by an agency of the United States Government or under a contract with an agency of the United States Government.					
<input type="checkbox"/> No.					
<input checked="" type="checkbox"/> Yes, the name of the U.S. Government agency and the Government contract number are: <u>NIH RO1 AI52453 and Vettrans Administration Merit Award</u>					

[Page 1 of 2]

Respectfully submitted,

SIGNATURE

TYPED or PRINTED NAME Colleen J McKiernanTELEPHONE 619-238-0999Date October 21, 2003REGISTRATION NO. 48,570

(if appropriate)

Docket Number: 6627-PR4043**USE ONLY FOR FILING A PROVISIONAL APPLICATION FOR PATENT**

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Docket Number 6627-PR4043

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Number 1 of 1

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## **HUMAN CATHELICIDIN ANTIMICROBIAL PEPTIDES**

### **BACKGROUND OF THE INVENTION**

Peptides with antimicrobial activity are found throughout nature and are known to be important in the defense of plants insects and animals. Peptides are present predominantly on skin and in the digestive tract as a preliminary form of defense. Many antimicrobial peptides display broad spectrum antimicrobial activity against both Gram-negative and Gram-positive bacteria, fungi and viruses. A number of families of antimicrobial peptides are listed in the attached figures and manuscript. Humans with inability to increase cathelicidins and defensins, two human antimicrobial peptides, in response to inflammatory stimuli develop atopic dermatitis. Genetic disorders such as Kostmann syndrome suffer from frequent infections and neutrophil dysfunction due to a deficiency in the production and processing of cathelicidins.

Most antimicrobial peptides are synthesized as inactive peptides that are activated by proteases to release active peptides. An inability to process the peptides results in disease. A lack of processing of alpha defensins from Paneth cells of Matrilysin deficient mice leads to impaired clearance of infection in the gut. Inhibition of the proteases that are involved in processing of the inactive peptides impairs bacterial clearance in wounds. Cleavage results in the generation of other active peptides that function to stimulate other processes such as chemotaxis and angiogenesis.

### **SUMMARY OF THE INVENTION**

The invention is the discovery of a series of active cathelicidin peptide fragments isolated from human sweat. The peptides are demonstrated to be fragments of cathelicidin by the use of antibodies and by amino acid sequencing of a portion of the peptides. The peptides have been demonstrated to have antimicrobial activity by antimicrobial assay

and low toxicity by a hemolysis assay. As the peptides are naturally occurring, the chance of adverse reaction to the peptides is minimal. The peptides have been shown to have substantially reduced pro-inflammatory activity known to be present in full length cathelicidin antimicrobial peptides. Moreover, the absence of resistant strains to these natural defenses of the body strongly suggests that mutations to create resistance to the antimicrobial peptides is detrimental to the viability of microbial pathogens.

The invention is the use of the antimicrobial peptides or fragments thereof isolated from sweat as antimicrobial agents for the prevention and/or treatment of infection in an organism including a human, other animal or plant. Preferably, the peptides are applied topically. In a human or other animal, the peptides can also be administered by other routes such as oral and parenteral. Additionally, the peptides can be used for decontamination of surfaces. The peptides may be used alone or in combination with each other. The peptides may be synthesized individually and combined. Alternatively, larger portions of the cathelicidins can be synthesized, treated with an appropriate protease to generate a mixture of antimicrobial peptides and administered to an organism or applied to a surface.

The invention is a method of activation of the antimicrobial peptides of the instant invention comprising incubation of the antimicrobial peptides in a bicarbonate buffer containing between about 15 mM to about 500 mM bicarbonate, preferably about 50 mM, preferably sodium bicarbonate. The bicarbonate buffer can also be used to modulate the solubility properties of the peptides for ease of application or administration.

The invention is further related to the compositions for use in the instant invention. The compositions include the peptides themselves as well as the peptides in the carbonate buffer of the invention.

The invention is a method for the identification of novel

antibacterial peptides comprising incubation of antimicrobial peptides with sweat for sufficient time to allow for proteolysis, isolation of peptide fragments and assaying for antimicrobial activity.

The invention is related to small molecule analogs or stabilized peptides based on the peptides of the invention or peptides identified by methods of the invention. Peptides can be modified for any of a number of reasons such as to improve pharmacokinetic and pharmacodynamic properties.

### **BRIEF DESCRIPTION OF THE DRAWINGS**

TABLE 1. Antimicrobial and hemolytic activity of human sweat peptides.

TABLE 2. Cathelicidin peptide antimicrobial function screen.

FIG. 1. Soluble antimicrobial activity on human skin.

FIG. 2. Serine protease in sweat enhances cathelicidin antimicrobial activity.

FIG. 3. Purification of novel cathelicidin peptides generated from LL-37.

FIG. 4. Identification of novel cathelicidin peptides generated from LL-37.

FIG. 5. Processing of LL-37 enhances antimicrobial activity.

FIG. 6. Processing of LL-37 decreases ability to stimulate keratinocyte IL-8

FIG. 7. Percent inhibition of Staph Aureus by LL-37 in 100 mM NaCl with or without bicarbonate buffer, 10% FCS- 2 hours to overnight.

FIG. 8. Growth of Staph Aureus in 100 mM NaCl, 10% FCS media containing various NaHCO<sub>3</sub> concentrations.

FIG. 9. Carbonate containing compounds increase the antimicrobial activity of LL-37.

FIG. 10. Bicarbonate itself, not pH, is responsible for antimicrobial enhancement.

FIG. 11. Bicarbonate enhances the activity of cathelicidin antimicrobial peptides.

FIG. 12. 25mM NaHCO<sub>3</sub> enhances LL-37 activity without effecting bacterial growth.

FIG 13. NaHCO<sub>3</sub> enhances antimicrobial activity against gram negative and gram positive bacteria.

FIG. 14. NaHCO<sub>3</sub> enables direct membrane disruption of *Staph aureus*.

FIG. 15. NaHCO<sub>3</sub> enhances the activity of various antimicrobial peptides.

FIG. 16. Electron microscopy reveals bacterial cell wall lysis by LL-37 and bicarbonate.

FIG. 17. Antimicrobial peptides and innate defense of the skin (40 pages)

FIG. 18. Antimicrobial peptides and innate defense of the skin (40 pages)

FIG. 19. Antimicrobial peptides and innate defense of the skin (37 pages)

The present invention will be better understood from the following detailed description of an exemplary embodiment of the invention, taken in conjunction with the accompanying drawings.

#### **DETAILED DESCRIPTION OF THE DRAWINGS**

FIG. 1. Soluble antimicrobial activity on human skin. Human sweat was concentrated 50X and separated by HPLC on C18. a) Absorbance profile at 214 nm for eluted material from 35% to 60% acetonitrile, inset: complete absorbance profile of eluted materials. b) Ability of material eluted in FIG. 1 a to inhibit growth of *S. aureus mprF* is shown as diameter of zone of *S. aureus mprF* inhibition. Several antimicrobial fractions were detected. Mass spectrometry identified 3 previously described antimicrobials; fractions

labeled (1) and (3) are dermcidin and DCD-1L respectively (MW 4701, MW 4818, respectively) confirmed by N-terminal sequencing ((1): SSLLEKGLDGA, (3): SSLLE,). (2): LL-37 identified by mass spectrometry (MW 4493) and immunoblot. Data representative of single experiment repeated 5 times with separate sweat preparations.

FIG. 2. Serine protease in sweat enhances cathelicidin antimicrobial activity. Antibacterial activity evaluated by radial diffusion assay against *S. aureus mprF* after incubation of LL-37 (32 $\mu$ M) in sweat. a) Increase in the inhibition zone is seen when incubated in sweat but not 10 mM PBS or distilled water (DW). b) Diameters of inhibition zone after 6hr incubation at 37°C in (SWT buffer; sweat buffer salts alone), (PBS), (D.W.; distilled water), (SWT; sterile filtered human sweat at 37°C), (SWT at 4°C), (SWT with P1, with protease inhibitor cocktail). c) Action of specific protease inhibitors on gain of antimicrobial activity. Data show increase in inhibition zone at 37°C after 6hr compared to 0 hr. Serine protease inhibitors AEBSF and Aprotinin were most effective. N.E.I. = neutrophil elastase inhibitor, L.E.I.= leukocyte elastase inhibitor. Data are triplicate determinations  $\pm$ SEM from single experiment representative of 3.

FIG. 3. Purification of novel cathelicidin peptides generated from LL-37 a) Human sweat was separated by HPLC on C18. Absorbance profile at 214 nm is shown for eluted material from 30% to 60% acetonitrile. Crude is sweat prior to addition of LL-37, overlay plots show separate runs of sweat following addition of 32nmol of LL-37 and incubation for 0 to 24 hr at 37°C. b) Antibacterial activity with radial diffusion assay against *S. aureus mprF* of fractions eluted from 24hr sample in a. Peaks with antimicrobial activity are labeled 1 and 2 and 3.

FIG. 4. Identification of novel cathelicidin peptides generated from LL-37. Following purification shown in Figure 3, major bioactive fractions were identified. a) peak (1) in Fig3a identified as KR-20 by mass spec. (MW 2468), and N-terminal sequence KRIVQRIKDVF, b) peak (2) detected 2 peptides; RK-31 and KS-30 (MW 3647, 3803, and RKSKEKTG, KSKEKTGK, respectively), c) Western blot analysis with anti-LL-37 was done on all



fractions from Fig 3, shown are fractions eluting at acetonitrile concentrations 46-55. Peaks labeled (2) and (3) in Figure 3 were immunoreactive. d) peak (3) identified as LL-37 (MW 4493). Data from single experiment representative of 3.

FIG. 5. Processing of LL-37 enhances antimicrobial activity. The antimicrobial activity of cathelicidin peptides was evaluated by radial diffusion assay against (a) *S. aureus*, (b) *E. coli*, (c) solution assay against *C. albicans* and (d) Group A Streptococci. The antimicrobial activity of each peptide was evaluated in 10%TSB/ 10mM phosphate buffer with several NaCl concentrations against *E.coli* (e) and *S.aureus* (f). Data shown are representative of triplicate determinations.

FIG. 6. Processing of LL-37 decreases ability to stimulate keratinocyte IL-8. Cathelicidin peptides were added to culture of normal human keratinocytes at a final concentration of 3 or 10  $\mu$ M then IL-8 release determined after 8 hr. All samples were endotoxin free by limulus assay. Data are mean  $\pm$ SEM of triplicate determinations.

FIG. 7. Percent inhibition of Staph Aureus by LL-37 in 100 mM NaCl with or without bicarbonate buffer, 10% FCS- 2 hours to overnight. Inhibition of growth of *S. aureus* by LL-37 with or without activation with carbonate anion was tested. As can be seen, the presence of 50mM  $\text{NaHCO}_2$  in the buffer substantially inhibited the growth of *S. aureus*.

FIG. 8. Growth of Staph Aureus in 100 mM NaCl, 10% FCS media containing various  $\text{NaHCO}_3$  concentrations. A number of concentrations of  $\text{NaHCO}_3$  were tested to determine the concentration required to activate the LL-37 without being toxic to the bacteria. As can be seen, killing of bacteria by LL-37 is greatly enhanced in the presence of 25 mM  $\text{NaHCO}_3$ . At higher concentrations (e.g., 100 mM  $\text{NaHCO}_3$ ) the effects of the bicarbonate on the activation of LL-37 are difficult to determine due to the apparent toxicity of the bicarbonate to the cells. Optimization of the concentration of bicarbonate to activate antimicrobial peptides is well within the ability of those skilled in the art.

FIG. 9. Carbonate containing compounds increase the antimicrobial

activity of LL-37. Carbonate was identified as the antimicrobial enhancing factor in mammalian tissue culture media via serial deletion of Minimal Essential Media (MEM) components. Staph aureus was cultured in 20% Tryptic Soy Broth (TSB), 10% Fetal Bovine Serum (FBS) and 70% of various media or media components with, or without, 32 $\mu$ M LL-37 to assess which components could increase antimicrobial activity. MEM buffers are 2200 mg/L NaHCO<sub>3</sub>, 140 mg/L NaH<sub>2</sub>PO<sub>4</sub> and 6800 mg/L NaCl. MEM salts are 200mg/L CaCl<sub>2</sub>, 400 mg/L KCl, 98 mg/L MgSO<sub>4</sub>. All solutions were adjusted to pH 7.4. Bacterial growth was measured as the change in turbidity at OD600 and % inhibition was determined by comparing bacterial growth in the presence of peptide to that in the absence of peptide. 32 $\mu$ M LL-37 inhibited Staph aureus growth poorly in TSB media alone, or with NaCl and FBS. In the presence of MEM buffers, inhibition was greatly increase and NaHCO<sub>3</sub>, was determined to be the buffering component responsible for this increase. This was confirmed with other carbonate containing compounds, 50mM KHCO<sub>3</sub> and Na<sub>2</sub>CO<sub>3</sub>, which also increased antimicrobial activity.

FIG. 10. Bicarbonate itself, not pH, is responsible for antimicrobial enhancement. Inhibition of Staph aureus was measured in 20% TSB, 10% FBS 1mM NaH<sub>2</sub>PO<sub>4</sub>, 150mM NaCl, with either 50mM NaHCO<sub>3</sub> or 50mM Glycine buffer at various pH. Only bicarbonate containing media was able to confer increased antimicrobial activity to LL-37.

FIG. 11. Bicarbonate enhances activity of cathelicidin antimicrobial peptides. Inhibition of Staph aureus growth in the presence of various concentrations of human (LL-37), murine (CRAMP) and porcine (PR-39) Cathelicidin antimicrobial peptides was measured in 20% TSB, 1mM NaH<sub>2</sub>PO<sub>4</sub>, with, or without, 50mM NaHCO<sub>3</sub> at pH 7.4. The presence of NaHCO<sub>3</sub> greatly reduced the minimal inhibitory concentration (MIC) of all three peptides.

FIG. 12. 25mM NaHCO<sub>3</sub> enhances LL-37 activity without effecting bacterial growth. The growth of Staph aureus in the presence, or absence, of 32  $\mu$ M LL-37 was measured in 20% TSB, 10% FBS, 1mM NaH<sub>2</sub>PO<sub>4</sub>, 150mM NaCl, and various concentrations of NaHCO<sub>3</sub>, all at pH 7.4. 25mM NaHCO<sub>3</sub>

yielded complete inhibition of bacterial growth in the presence of LL-37, but not without peptide. High concentrations of  $\text{NaHCO}_3$  retarded the growth of *Staph aureus*.

FIG. 13.  $\text{NaHCO}_3$  enhances antimicrobial activity against gram negative and positive bacteria. *Staph aureus* and *E. coli* were resistant to  $16\mu\text{M}$  CRAMP without  $50\text{mM}$   $\text{NaHCO}_3$ , but became susceptible in its presence in 20% TSB,  $1\text{mM}$   $\text{NaH}_2\text{PO}_4$  at pH 7.4. Group B Strep (GBS) and *Salmonella* were susceptible even without  $\text{NaHCO}_3$ .

FIG. 14.  $\text{NaHCO}_3$  enables direct membrane disruption of *Staph aureus*. Release of cytoplasmic expressed beta-galactosidase was measure from bacteria with and without the addition of  $\text{NaHCO}_3$ . A) polymyxin B induces greater leakage of cytoplasmic protein with carbonate. B) LL-37 induces greater leakage of cytoplasmic protein with carbonate

FIG. 15.  $\text{NaHCO}_3$  enhances the activity of various antimicrobial peptides. The ability of  $50\text{mM}$   $\text{NaHCO}_3$  (EF) to enhance the activity of diverse antimicrobial peptides against *Staph aureus* was tested in 20% TSB, 10% FBS,  $1\text{mM}$   $\text{NaH}_2\text{PO}_4$ ,  $150\text{mM}$   $\text{NaCl}$ , pH 7.4. The activity of all 3 Cathelicidins, Cryptdin-4 and Human Beta Defensin-2 (HBD-2) were enhanced by the addition of bicarbonate. Several other antimicrobial peptides were not enhanced.

FIG. 16. Electron microscopy reveals bacterial cell wall lysis by LL-37 and bicarbonate. *Staph aureus* was grown for four hours in 20% TSB,  $1\text{mM}$   $\text{NaH}_2\text{PO}_4$  with or without  $25\text{mM}$   $\text{NaHCO}_3$  or  $32\mu\text{M}$  LL-37. Bacteria were fixed in Karnovsky's Solution 24 hours and processed for EM on a Zeiss EM 10B Transmission Electron Microscope by the VA Microscopy Core Facility. The presence of  $25\text{mM}$   $\text{NaHCO}_3$  alone had no visible effect on the bacterial cell wall.  $32\mu\text{M}$  LL-37 caused a small amount of damage to bacterial cell walls in the absence of  $\text{NaHCO}_3$ , but totally ablated them in its presence, causing cell lysis. Mag 42,000X, insets are 110,000X.

Although an exemplary embodiment of the invention has been described above by way of example only, it will be understood by those

skilled in the field that modifications may be made to the disclosed embodiment without departing from the scope of the invention, which is defined by the appended claims.

**WE CLAIM:**

## **CLAIMS**

1. A method of prevention or treatment microbial infection in an organism comprising administration of an antimicrobial peptide or fragment thereof to the organism.

2. The method of claim 1, wherein the microbial infection is caused by an organism selected from the group consisting of gram positive bacteria, gram negative bacteria, fungus and virus.

3. The method of claim 1, wherein the antimicrobial peptide or fragment thereof is selected from the group consisting of bombinins, cecropins, dermaseptins magainins, defensins, protegrins and cathelicidins.

4. The method of claim 1, wherein the antimicrobial peptide or fragment thereof is a cathelicidin.

5. The method of claim 4, wherein the fragment of cathelicidin comprises a peptide of at least 4 amino acids in length.

6. The method of claim 4 wherein the fragment of cathelicidin comprises a peptide of at least 4 amino acids in length from a mature amp domain of the cathelicidin.

7. The method of claim 4, wherein the fragment of cathelicidin is selected from the group consisting of LL-37, LL-25, LL-20, LL-15, LL-12, GD-23, RK-19, EK-20, VQ-17, EK-15, GK-17, EF-15, D-EK-20, RRR-20, PR-39, KR-20, RK-30, RK-31, KS-30, DCD-1L, and DCD.

8. The method of claim 4, wherein the fragment of cathelicidin is generated by incubation of substantially a full length cathelicidin with

protease.

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9. The method of claim 4, wherein the fragment of cathelicidin is generated by incubation of substantially a cathelicidin amp fragment with protease.

10. The method of claim 1, wherein the antimicrobial peptide or fragment thereof is administered by topical application, oral or parenteral routes of administration.

11. The method of claim 1, wherein the antimicrobial peptide of fragment thereof is combined with a bicarbonate buffer comprising about 15 mM to 500 mM bicarbonate before administration.

12. A method of enhancing activity of an antimicrobial peptide or fragments thereof comprising incubation of the antimicrobial peptides in a bicarbonate buffer system wherein the buffer system comprises a bicarbonate concentration between about 15 mM and 500 mM bicarbonate.

13. The method of claim 12, wherein the antimicrobial peptide or fragment thereof is selected from the group consisting of bombinins, cecropins, dermaseptins magainins, defensins, protegrins and cathelicidins.

14. A composition of matter comprising an antimicrobial peptide or fragment thereof in a bicarbonate buffer system wherein the buffer system comprises a bicarbonate concentration between about 15 mM and about 500 mM bicarbonate.

15. The composition of claim 14, wherein the antimicrobial peptide

or fragment thereof is selected from the group consisting of bombinins,  
cecropins, dermaseptins magainins, defensins, protegrins and  
cathelicidins.

16. A method of decontamination of a surface comprising  
application of an antimicrobial peptide or fragment thereof to the surface.

17. A method for the identification of novel antimicrobial peptides  
comprising, incubation of antimicrobial peptides with sweat for sufficient  
time to allow for proteolysis, isolation of peptide fragments and assaying  
for antimicrobial activity.

## **ABSTRACT**

The production of antimicrobial peptides is essential for the defense against infection. The invention is the discovery of a number of antimicrobial peptides in human sweat. The invention is a method of prevention and treatment of microbial infections comprising the use of the peptides. The invention is a method of activation of microbial peptides comprising incubation with bicarbonate buffer. The invention is a method for the discovery of new antimicrobial peptides comprising incubation of antimicrobial peptides with sweat, isolation of antimicrobial peptide fragments and testing them for the presence of antimicrobial activity. The invention is the development and use of peptide analogs of the antimicrobial peptides disclosed in the application or identified using the method of the invention.



# **Post-secretory processing generates multiple cathelicidins for enhanced topical antimicrobial defense<sup>1</sup>**

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**Abstract:**

The production of antimicrobial peptides is essential for defense against infection. Many of the known human antimicrobial peptides are multifunctional, with stimulatory activities such as chemotaxis while simultaneously acting as natural antibiotics. In humans, eccrine appendages express DCD and CAMP, genes encoding proteins processed into the antimicrobial peptides Dermcidin and LL-37. Here we show that after secretion onto the skin surface, the CAMP gene product is processed by a serine protease-dependent mechanism into multiple antimicrobial peptides previously unknown and distinct from the cathelicidin LL-37. These peptides show enhanced antimicrobial action, acquiring the ability to kill skin pathogens such as *Staphylococcus aureus* and *Candida albicans*. Furthermore, although LL-37 may influence the host inflammatory response by stimulating IL-8 release from keratinocytes, this activity is lost in subsequently processed peptides. Thus, a single gene product encoding an important defense molecule alters structure and function in the topical environment to shift the balance of activity towards direct inhibition of microbial colonization.

**Introduction:**

Peptides with antimicrobial activity are found throughout nature and are known to be important for the immune defense of plants, insects and animals(1). Many of these peptides exhibit a broad spectrum of antimicrobial activity, inhibiting or killing Gram-positive and Gram-negative bacteria, fungi and viruses. In mammals, antimicrobial peptides belonging to the cathelicidin family have been shown to be important for the antimicrobial efficacy of neutrophils, macrophages, and mast cells (2-6). In addition, epithelia of lung, gut, urinary bladder, oral mucosa and skin produce antimicrobial peptides of the defensin and cathelicidin family constitutively or in response to injury (7-9). At the epithelial interface with the external environment, these molecules are thought to serve as a rapid first line defense for inhibition of microbial proliferation and invasion.

In mammalian skin, cathelicidins have been directly shown to be essential for defense against invasive bacterial infection by Group A Streptococcus(10). Found in abundance in neutrophils and mast cells, the cathelicidins are expressed at relatively low levels in normal keratinocytes but are rapidly induced during inflammation(11). The recruitment of cathelicidin-rich cells, and increase in expression by the epithelial keratinocytes, leads to accumulation of cathelicidins in wound fluid and in the overlying crust(12). Recently, cathelicidins and dermcidin have also been found constitutively produced by the eccrine apparatus and secreted constitutively into human sweat (13, 14). In this scenario, antimicrobial activity becomes available at the most external interface, providing an inhibitory barrier to infection.

The clinical consequences of antimicrobial peptide expression are seen by observations that patients with atopic dermatitis lack the ability to increase cathelicidins and defensins in response to inflammatory stimuli(15). Since patients with atopic dermatitis are uniquely susceptible to infection when compared to normal individuals or those with elevated expression as seen in psoriasis, this first line immune defense mechanism appears to have an essential function for resistance against skin infections. Similarly, patients with Kostmann syndrome, a rare inherited disorder characterized by frequent infections and neutrophil dysfunction, have a deficiency in production and processing of

cathelicidin (16). Such emerging clinical associations support the need to further explore the function and regulation of this evolutionarily ancient aspect of the human immune system.

Most antimicrobial peptides are synthesized as inactive preproproteins that require enzymatic processing for release of active peptides. Lack of processing, such as seen in alpha defensins from Paneth cells of Matrilysin deficient mice(17), leads to impaired clearance of infection in the gut. Similarly, inhibition of processing of porcine cathelicidins by elastase impairs bacterial clearance in wounds(18). The enzymatic processing of cathelicidins from a pro-protein to a two-component solution consisting of the cathelin prodomain and the C-terminal cationic antimicrobial peptide, LL-37, can have multiple consequences for immune defense. The cathelin domain is itself antimicrobial and functions to inhibit cysteine proteases such as Cathepsin L (19). LL-37, while functional as a broad spectrum antimicrobial, can also stimulate chemotaxis and angiogenesis by binding formyl peptide receptor-like-1 (FPR1) (20, 21) and improve reepithelialization to enhance wound repair(22).

In this study we sought to determine if additional antimicrobial peptides are present in the most superficial barrier topically provided by human sweat. Our findings suggest that human cathelicidin is further processed and enables a shift in biological activity towards antimicrobial function and away from the ability to stimulate host response.

## Materials and Methods

### *Sweat collection and processing*

Sweat was collected on paper tissues (Kimwipes, Kimberly-Clark, GA) from healthy volunteers after exercise as previously described(13). After collection, 20 ml of sweat was centrifuged at 2000g for 15 min. at 4°C, filtered through a 0.20 µl filter (Acrodisc syringe filter, 0.2 µm, low protein binding, Fisher scientific, Tustin, CA), and frozen at -80°C. For some experiments sweat was lyophilized to dryness, then suspended in 400 µl of distilled water (DW: cell culture grade, endotoxin free, GibcoBRL, Grand island, NY). For analysis of LL37 processing by sweat, 1.6 nmol of LL-37 synthetic peptide was incubated in 50 µl of sweat for 0, 1, 6, 24 hrs at 37 °C or 4°C. In some experiments proteinase inhibitors including; mixed protease inhibitor cocktail (1tab/10ml, Roche, Indianapolis, IN), 100 µg/ml Bestatin, 10 µg/ml E-64, 10 µg/ml Aprotinin, (Sigma, St Louis, MO); 100 µM AEBSF, 100 µM Neutrophil elastase inhibitor (NEI) or 100 µM leukocyte elastase inhibitor (LEI) (Calbiochem, San Diego, CA) were added during incubation. After incubation, 2 µl was assayed by radial diffusion assay to determine antibacterial activity. For analysis by HPLC 32 nmoles LL-37 was incubated in 100ul. To control for potential contamination eluted from paper tissues, parallel processing was done on tissues soaked in phosphate-buffered saline (PBS:137 mM NaCl, 2.7 mM KCL, 4.3 mM Na<sub>2</sub>HPO<sub>4</sub>-H<sub>2</sub>O, 1.4 mM KH<sub>2</sub>PO<sub>4</sub>, pH 7.4). No antimicrobial activity was detectable in these preparations. Protein concentrations were evaluated by BCA assay (protein assay reagent, Pierce, Rockford, IL) or Bradford protein assay (Bio-Rad, Hercules, CA) according to manufacture's instructions. Human tissue and blood collection was approved by the UCSD Human Research Protection Program.

### *Peptide synthesis*

Dermcidin, LL-37, RK-31, KS-30, and KR-20 peptides were commercially prepared by Synpep Corporation, Dublin, OR. Peptide amino acid sequences were LLGDFFRKSKEKIGKEFKRIVQRIKDFLRNLPRTES (LL-37), RKSKEKIGKEFKRIVQRIKDFLRNLPRTES (RK-31), KSKEKIGKEFKRIVQRIKDFLRNLPRTES (KS-30), KRIVQRIKDFLRNLPRTES (KR-20), SSLLKGLDGAKKAVGGLGKLGKDAVEDLESVGKGAVHDVKDVLDSV (dermcidin). All

synthetic peptides were purified by HPLC and identity confirmed by mass spectrometry.

#### *HPLC chromatography*

Peptide separation was performed using an AKTA purification system (Amersham Pharmacia Biotech, Piscataway, NJ) on a Sephasil peptide C18 column (12  $\mu$ m, ST 4.6/250, Amersham Pharmacia Biotech, Piscataway, NJ). Concentrated human sweat, or LL-37 incubated in sweat, was separated by reversed phase (RP-HPLC) following column equilibration in 0.1% TFA at a flow rate of 0.5 ml/min and eluted using gradients of 0-35% and 35-60% acetonitrile for 16 min, 67 min, or 0-60% acetonitrile for 120 min. Column effluent was monitored at 214, 230, and 280nm. All collected fractions (1 ml) were lyophilized and suspended in 10  $\mu$ l of DW for antimicrobial radial diffusion assay.

#### *Western and Immunoblot analysis*

Fractions purified by HPLC as described above were evaluated by quantitative dot-blot and Western Blot. 2  $\mu$ l of each fraction was compared to a standard curve of synthetic LL37 peptide applied onto PVDF membrane (Immobilon-P, Millipore, MA). Antibody used was rabbit anti-LL-37 polyclonal antibody derived and affinity purified against the entire LL-37 peptide. For immunoblot, membranes were blocked (0.1% TTBS: 5% nonfat milk in 0.1% Tween 20 / tris buffered saline (TBS: 150 mM NaCl, 10 mM Tris Base, pH 7.4)) for 60 min at R.T., and then rabbit anti LL37 polyclonal antibody (1:5000 in blocking solution) was incubated with the membrane overnight at 4°C. After washing 3x with 0.1% TTBS, horseradish peroxidase labeled goat anti rabbit antibody (1:5000 in the blocking solution, (DAKO, CA) was incubated with the membrane for 60 min, R.T. After washing the membrane again with 0.1% TTBS, the membrane was immersed in ECL solution (Western Lightning Chemiluminescence Reagents Plus, New Lifescience Products, Boston, MA) for 60 sec then exposed to X-ray film (Kodak). For, Western-blot analysis, sweat samples (10  $\mu$ l) were separated by 16.5% tris-tricine / peptide gel (BIO-RAD, Hercules, CA), and then transferred on to a PVDF membrane (Immobilon-P, Millipore, MA). For positive control, 5 pmol LL-37 synthetic peptide was applied.

#### *Mass spectrometry and Protein sequence analysis*

Mass spectrometry was performed by Center for Mass Spectrometry, The Scripps Research Institute

(La Jolla, CA). MALDI-MS spectra were obtained with a Voyager DE-RP MALDI-TOF mass spectrometer (PerSeptive Biosystems, Framingham, MA) equipped with a nitrogen laser (337 nm, 3-ns pulse). Spectra were collected in reflector mode. The accelerating voltage in the ion source was 20 kV. Data were acquired with a transient recorder with 2-ns resolution. The matrix used in this work was -cyano-4-hydroxycinnamic acid dissolved in water/acetonitrile (1:1, v/v) to give a saturated solution at room temperature. To prepare the sample for analysis, 1  $\mu$ l of the peptide solution (containing 1-10 pmol of protein in 0.1% trifluoroacetic acid) was added to 1  $\mu$ l of the matrix solution and applied to a stainless steel sample plate. The mixture was then allowed to air dry on the sample plate before being introduced into the mass spectrometer. Each spectrum was produced by accumulating data using 128 laser pulses. Mass assignments were assigned with an accuracy of approximately  $\pm 0.1\%$  ( $\pm 1$  Da/1000 Da). Protein sequence analysis for target HPLC fractions was performed by Division of Biology Protein Sequencer Facility, University of California, San Diego. The amino acid sequencing was performed on an Applied Biosystems Procise Model 494 sequencer using the "pulsed-liquid" program supplied by the manufacture.

#### *Antimicrobial assays*

For screening of antimicrobial activity of HPLC fractions, radial diffusion assay was used as previously described(13). Lyophilized HPLC fractions were dissolved in DW (GibcoBRL, Grand Island, NY), and tested against *Staphylococcus aureus mprF*. Thin plates (1 mm) of 1% agarose in 0.5% tryptone containing approximately  $1 \times 10^6$  cells/ml of *S. aureus mprF* were used. 1 mm wells were punched in the plates and 2 $\mu$ l of samples dissolved in tissue culture grade sterile water were loaded in each well. As a positive control, synthetic LL-37 was applied to separate wells. After incubation at 37 °C overnight, the inhibition zones were recorded by CCD camera and diameters measured.

To evaluate antimicrobial activity against wild-type *S. aureus* (clinical isolate), and enteroinvasive *E.coli* O29, both radial diffusion and solution killing assays were done. Radial diffusion assays were done as described for *S.aureus mprF*. Solution killing was done in 10%TSB in 10mM PB (TSB= 30gm/L Tryptic Soy Broth, Sigma, St Louis MO; 20X PB= 27.6 g/L NaH<sub>2</sub>PO<sub>4</sub>-H<sub>2</sub>O, 53.65 g/L Na<sub>2</sub>HPO<sub>4</sub>-7H<sub>2</sub>O, pH 7.4). Bacteria in log-phase growth were suspended to  $1 \times 10^6$  cells/ml, peptide

added and incubated at 37°C for 2 hr. Bacteria were then plated on TSB agar (TSB, Bactoagar 13g/L, Becton Dickinson, Sparks MD) for direct colony count and determination of CFU. Activity against Group A Streptococcus (NZ131) was done only in solution assay as described. To evaluate antimicrobial activity in high salt conditions, solution assay was done in 10%TSB/10mM PBS with several salt concentrations (concentration of NaCl; 10, 50, 100, 150, 300, 500 mM). Action against the *C. albicans* was determined in Dixon medium (0.6% Peptone, 4% Malt Extract, 1% Glucose, 0.1% Ox Bile, 1% Tween-80) in sterile 96-well microtiter plates (Corning Inc., Corning, NY) at a final volume of 50 µl. The assay mixtures contained  $1-2.5 \times 10^4$  CFU/ml freshly grown *Candida*, 20% Dixon medium, 0.6mM phosphate buffer pH 7, and 16 µg/ml chloramphenicol. Microtiter plates were incubated at 37°C for 24h with peptides, then plated on Dixon agar to determine the MFC (minimum fungicide concentration).

#### Hemolysis Assay

Hemolytic activity was determined on human whole blood. Freshly obtained whole blood was washed 3X in PBS and resuspended in PBS at its original volume containing peptides at indicated concentrations. Samples were incubated at 37 °C for 1.5 to 3 h, and hemolysis determined by centrifuging at 300g and measurement of the absorbance of the supernatant at 578 nm. Hemolytic activity of each peptide was expressed as the percentage of total hemoglobin released compared to that released by incubating with 0.1% Triton X-100.

#### *Measurement of IL-8 release from keratinocytes*

Normal human keratinocytes (NHK) were cultured in EpiLife cell medium (Cascade Biologics, Portland, OR) containing 0.06 mM  $\text{Ca}^{2+}$ , 1x EpiLife-defined growth supplement (EDGS), 50 U/ml penicillin and 50 µg/ml streptomycin (Invitrogen, Carlsbad, CA). Keratinocytes were seeded in a 96-well plate and grown to confluence under standard tissue culture conditions. Cells were incubated with 3µM or 10µM LL-37 or LL37-derived peptides for 8 hours at 37°C. Supernatants were collected and stored at -20°C overnight. IL-8 ELISA Assay was performed according to the manufacturer's instructions (BD OptEIA, Pharmingen, San Diego, CA). Supernatants were diluted 1:10 for assay.



Simultaneously, LDH assays (Roche, Indianapolis, IN) were used to assess the cytotoxicity of peptides to keratinocytes. LDH release following peptide exposure was compared to release induced by 1% Triton X100.

## Results

To identify antimicrobial activity present at the skin surface, human sweat was collected from normal volunteers, concentrated, and separated by high performance liquid chromatography (HPLC). Fractions of the material eluted between 35-65% acetonitrile were individually evaluated for the ability to inhibit the growth of *S. aureus mprF* by radial diffusion assay (Figure 1). Multiple distinct fractions were found to be active in this assay. Prior evaluations of human sweat have shown that the antimicrobial peptides LL-37 and dermcidin are produced by the eccrine apparatus and secreted into the topical soluble environment of sweat. As expected, these molecules were detectable in the sweat preparation shown in Figure 1. The presence of LL-37 was confirmed by immunoblot analysis with antibody specific to LL-37 and by MALDI-TOF mass spectrometry. The dermcidin peptides, DCD and DCD-1L, were identified by MALDI-TOF mass spectrometry and N-terminal amino acid sequencing.

Immunoblot analysis with anti-LL-37 antibody of all fractions isolated by HPLC from the human sweat suggested that other molecules related to LL-37 may be present in fractions eluting between 43 and 48% acetonitrile. These fractions were associated with antimicrobial activity but not identifiable by MALDI-TOF mass spectrometry and N-terminal amino acid sequencing from the concentrated sweat preparations. Based on the immunoreactivity and elution profile we hypothesized that these antimicrobial molecules were alternative forms of LL-37 that were further processed in sweat to unique cathelicidin peptides. To test this, human sweat was freshly collected, sterilely filtered, then synthetic LL-37 added to a final concentration of 32  $\mu$ M. The relative ability of this solution to inhibit the growth of *S. aureus mprF* was then evaluated. Following incubation at 37°C in sweat, LL-37 increased the zone of inhibition and apparent antimicrobial activity (Figure 2a). Incubation of LL-37 under identical conditions in PBS or distilled water did not affect activity. This increase in apparent antimicrobial activity did not occur at 4°C and was inhibited by the addition of protease inhibitor cocktail, thus suggesting the gain in antimicrobial function was a consequence of an enzymatic process (Figure 2b). Addition of specific inhibitors of potential processing enzymes demonstrated that the serine protease inhibitors AEBSF and Aprotinin were most effective in blocking the increase in antimicrobial activity generated by incubation of LL-37 in sweat (Figure 2c).

An increase in the relative ability of LL-37 to inhibit growth of *S. aureus mprF* suggested that the enzymatic processing of this peptide resulted in alternative forms with either increased direct antimicrobial activity or an ability to synergize with the parent peptide. To identify these processed forms of LL-37, synthetic peptide was incubated with the sterile sweat preparation and separated by HPLC after various periods of incubation (Figure 3a). An increase in the relative abundance of several proteins was seen over time. Correlation of this profile with antimicrobial activity showed fractions eluting at 39% and 48% acetonitrile gained antimicrobial activity coincident with an increase in relative abundance of peptides eluting at these positions (Figure 3b). A third major peak of antimicrobial activity seen between 53 and 56% acetonitrile showed a relative decrease in abundance as estimated by absorbance at 214 nm. MALDI-TOF mass spectrometry and N-terminal sequencing of the peptide eluting at 39% acetonitrile identified this as a 20 amino acid cathelicidin derivative, KR-20 (Figure 4a). Similar analysis of peptides eluting at 48% acetonitrile identified two additional cathelicidin peptides RK-31 and KS-30 (Figure 4b). Western blot analysis with antibody against LL-37 confirmed antigenic similarity of RK-31 and KS-30 to parent LL-37 (Figure 4c). KR-20 was not detectable with this antibody (data not shown). Material eluting at 55-56% acetonitrile and decreasing in abundance with incubation was identified as LL-37 by both Western blot and Mass spec analysis (Figure 4c & d).

The newly described human cathelicidin peptides KR-20, RK-31 and KS-30 eluted at positions corresponding to unidentified antimicrobial activity seen in crude sweat preparations partially purified in Figure 1. The low relative abundance of these peptides, yet easily detectable antimicrobial activity suggested that these peptides might gain antimicrobial activity with processing when compared to LL-37. To compare the antimicrobial activity of these cathelicidins, purified synthetic peptides corresponding to LL-37, RK-31, KS-30 and KR-20 were assessed by both radial diffusion assay and solution assay against a variety of microbes (Figure 5). RK-31 and KS-30 showed greatly increased action against wild-type *S. aureus* and *E. coli*. And all three new peptides showed increased fungicidal activity against *Candida albicans*. Furthermore, these peptides were synergistic, killing bacteria at lower concentrations when present together, (Figure 5d), and maintained activity at increased salt

conditions (Figure 5e,f). Hemolytic activity against human erythrocytes was minimal as seen by assay at a concentration 5 to 10 times greater than that required for antimicrobial activity (Table 1). However, despite gaining antimicrobial activity by processing to shorter forms of the cathelicidin peptide, hemolytic activity of these antimicrobials decreased relative to LL-37.

In addition to the function of antimicrobial peptides as natural antibiotics, many of these molecules have been associated with the ability to stimulate a variety of host responses. To determine if the secretion and processing of cathelicidin peptides at the surface of the skin could also stimulate a host inflammatory response, human keratinocytes were grown in culture and assayed for the release of IL-8 in response to these peptides. All peptide solutions were endotoxin free. LL-37 had a potent ability to stimulate IL-8 release from keratinocytes, but processing to the shorter form of these cathelicidins decreased the ability to stimulate IL-8 (Figure 6). Cellular toxicity of these peptides was simultaneously evaluated by determination of the release of lactate dehydrogenase into keratinocyte culture medium. At a concentration of 3  $\mu$ M none of the peptides evaluated significantly increased the permeability of keratinocyte membranes (data not shown).

## Discussion

Synthesis, processing and release of antimicrobial peptides are essential elements for defense against infection. In epithelia such as skin, oral mucosa, lung and gut, the release of antimicrobial peptides appears to play a particularly important defensive role. However, the expression of antimicrobial peptides by normal skin, an epithelial barrier exposed to constant microbial challenge, is relatively low when compared to mucosal epithelia of other organs. Partial explanation for this apparent inconsistency is found in recent observations that the antimicrobial peptides hCAP 18/LL-37 and dermcidin are produced by eccrine glands and secreted onto the surface of the skin in sweat. This system would provide a mechanism by which a constitutive antimicrobial barrier may form at the skin surface above the permeability barrier of the stratum corneum and in direct contact with the external environment. A problem with this model is that Dermcidin and LL-37 have poor ability to inhibit the growth of important skin pathogens such as *S. aureus* and *Candida albicans*. Because of this limitation, we hypothesized that sweat contains additional antimicrobial peptides with increased activity against relevant skin pathogens. In this study we show that enhanced antimicrobial activity exists in normal human sweat, and is the consequence of processing of LL-37 to previously unknown naturally occurring cathelicidin peptides.

HPLC separation of concentrated sweat samples from different individuals showed remarkable consistency in the elution profile of antimicrobial molecules. Screening assays were done with *S. aureus* mprF, a mutant that lacks the ability to modify anionic membrane lipids with L-lysine(23). This mutation leads to an increase in surface anionic charge and greater binding by cationic host defense molecules. Thus, this strain of *S. aureus* amplifies sensitivity to cationic antimicrobial peptides. Initial purifications confirmed the sensitivity of this approach by detecting peptides already known to occur in human sweat such as LL-37, DCD and its variant DCD-1L. However, antimicrobial activity associated with less abundant peptides in the crude human sweat preparation was difficult to purify by standard biochemical approaches. Detection of these fractions by antibody against LL-37 suggested that some of these less abundant yet apparently potent antimicrobial molecules were similar to LL-37.

Analysis of antimicrobial activity and identification of new peptides generated from synthetic

LL-37 showed that further processing occurs by a serine protease present in sweat. These previously unknown peptides eluted at positions similar to the antimicrobial activity detected in native sweat, suggesting that RK-31, KS-30 and KR-20 are naturally occurring, but less abundant in sweat immediately after secretion. Unlike the techniques used here to collect sweat for study, under normal conditions sweat remains at the skin surface for further processing and concentration by evaporation. This final enzymatic modification of cathelicidins is thus distinct from that previously studied during neutrophil activation. Bovine and porcine precursor cathelicidins in specific granules are acted upon by elastase in the azurophilic granule to release a single C-terminal antimicrobial peptide(24). In humans, the full-length cathelicidin hCAP-18 can be cleaved to LL-37 by proteinase 3, a protease present in human neutrophils(25). In the vagina at low pH, another serum protease, gastricsin, processes a slightly longer C-terminal peptide (ALL-38) from hCAP-18(26). These systems have not shown further processing of the C-terminal peptide. This may be due to lack of detection of these peptides in prior studies or due to the presence of additional serine proteases at the skin's surface, such as activated tryptases and kallikrein(27). These and/or other serine proteases of host origin, as well as serine proteases potentially released by microflora on the skin, may be responsible for the processing observed in the study. Since this processing leads to an important gain in antimicrobial function and modification of host stimulatory effects by LL-37, activity of these enzymes, their expression, and balance with known skin serine protease inhibitors such as bikunin(28), elafin(29), anti-leukoprotease(30), and plasminogen activator inhibitor-2 (31), take on new found importance for understanding immune defense.

The gain in antimicrobial activity observed following processing of LL-37 to RK-31 and KS-30 was unexpected. Structural analysis of LL-37 has shown it is an antipathetic alpha-helical molecule that probably kills by ionic association with the membrane and subsequent disruption of the lipid bilayer through formation of a toroidal pore. Change in the net charge by processing is an unlikely explanation for the increase in activity since the estimated PI of LL-37 is 10.4, identical to that of KS-30. Cleavage of the 6 amino acids at the amino terminus representing non-polar, polar and acidic residues leads to exposure of a basic residue that may contribute to the increase in activity. Previous analysis of the structurally distinct cathelicidin PR-39 has shown that N-terminal lysines are important

to activity and may function by facilitating initial ionic interaction with the anionic microbial surface (32). This explanation is likely incomplete since one of the largest gains in function seen by processing of LL-37 to shorter peptides was in its ability to kill wild-type *S.aureus*, an organism that has developed apparent resistance to cationic antimicrobial peptides such as beta defensins and LL-37 by modification of charge at the cell surface. Furthermore, structural modifications of defensins have major effects on chemotactic activity and lesser influence on antibacterial function (33), a phenomenon similar to that seen here with the loss of IL-8 stimulatory capacity. Additional high resolution structural studies of LL-37 and its shorter more active related peptides are necessary and may yield important new information relevant to the mechanism of action.

Understanding the role of antimicrobial peptides in mammalian immunity is complicated by many observations that these peptides also act on the host to stimulate a variety of important responses related to defense against injury. Cathelicidins were first found in mammalian skin due to their ability to increase fibroblast proteoglycan synthesis(34). Defensins and cathelicidins have both been shown to have chemotactic activity, possibly functioning through specific receptors such as CCR6 or FPRL-1 respectively(21, 35). Consistent with these prior observations, LL-37 was found in the present study to be a potent stimulus for IL-8 release from cultured keratinocytes. This effect was diminished upon processing to RK-31 or KS-30, and almost completely eliminated in the shortest KR-20 cathelicidin. It is not clear if topical LL-37 secreted into sweat would have a similar affect *in vivo* on epidermal keratinocytes when separated by the formidable barrier of the stratum corneum. However, the inhibition of pro-inflammatory functions by LL-37 in normal epithelia would be a beneficial mechanism to regulate unintended inflammation.

The present findings show post-secretory processing of LL-37 occurs at the skin surface. Generation of additional potent antimicrobial peptides suggests a model in which a single gene of the innate defense system can generate multiple differentially active products. Cathelicidins found in mammalian species such as the cow, pig, sheep, and horse are found as multiple copies encoding distinct C-terminal peptides. Conversely, in man and rodents, only a single cathelicidin gene product is known. Following initial processing of the pro-protein into the cathelin-like domain and LL-37, two

distinct defense molecules are activated; the cathelin-like domain possessing both antimicrobial activity and an ability to act as a protease inhibitor, and LL-37 having antimicrobial function combined with a range of host stimulatory capacities. The present findings suggest that in humans, further cathelicidin diversity is generated by post-secretory processing, tipping the balance of function towards antimicrobial action and away from effects on the host. Thus, the single human cathelicidin gene generates multiple products with a range of biological activities, each relevant to the local environment in which they are released.



All citations incorporated by reference 16

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## Figure Legends

### Figure 1.

#### Soluble antimicrobial activity on human skin

Human sweat was concentrated 50X and separated by HPLC on C18. a) Absorbance profile at 214 nm for eluted material from 35% to 60% acetonitrile, inset: complete absorbance profile of eluted materials. b) Ability of material eluted in Fig 1a to inhibit growth of *S. aureus mprF* is shown as diameter of zone of *S. aureus mprF* inhibition. Several antimicrobial fractions were detected. Mass spectrometry identified 3 previously described antimicrobials; fractions labeled (1) and (3) are dermcidin and DCD-1L respectively (MW 4701, MW 4818, respectively) confirmed by N-terminal sequencing ((1): SSLLEKGLDGA, (3): SSLLE, ). (2): LL-37 identified by mass spectrometry (MW 4493) and immunoblot. Data representative of single experiment repeated 5 times with separate sweat preparations.

### Figure 2.

#### Serine protease in sweat enhances cathelicidin antimicrobial activity

Antibacterial activity evaluated by radial diffusion assay against *S. aureus mprF* after incubation of LL-37 (32uM) in sweat. a) Increase in the inhibition zone is seen when incubated in sweat but not 10 mM PBS or distilled water (DW). b) Diameters of inhibition zone after 6hr incubation at 37°C in (SWT buffer; sweat buffer salts alone), (PBS), (D.W.; distilled water), (SWT; sterile filtered human sweat at 37°C), (SWT at 4°C), (SWT with PI, with protease inhibitor cocktail). c) Action of specific protease inhibitors on gain of antimicrobial activity. Data show increase in inhibition zone at 37°C after 6hr compared to 0hr. Serine protease inhibitors AEBSF and Aprotinin were most effective. N.E.I. = neutrophil elastase inhibitor, L.E.I.= leukocyte elastase inhibitor. Data are triplicate determinations ±SEM from single experiment representative of 3.

Figure 3.

**Purification of novel cathelicidin peptides generated from LL-37**

- a) Human sweat was separated by HPLC on C18. Absorbance profile at 214 nm is shown for eluted material from 30% to 60% acetonitrile. Crude is sweat prior to addition of LL-37, overlay plots show separate runs of sweat following addition of 32nmols of LL-37 and incubation for 0 to 24 hr at 37°C.
- b) Antibacterial activity with radial diffusion assay against *S. aureus mprF* of fractions eluted from 24hr sample in a. Peaks with antimicrobial activity are labeled 1 and 2 and 3.

Figure 4.

**Identification of novel cathelicidin peptides generated from LL-37**

Following purification shown in Figure 3, major bioactive fractions were identified. a) peak (1) in Fig3a identified as KR-20 by mass spec. (MW 2468), and N-terminal sequence KRIVQRIKDVF, b) peak (2) detected 2 peptides; RK-31 and KS-30 (MW 3647, 3803, and RKSKEKIG, KSKEKIGK, respectively), c) Western blot analysis with anti-LL-37 was done on all fractions from Fig 3, shown are fractions eluting at acetonitrile concentrations 46-55. Peaks labeled (2) and (3) in Figure 3 were immunoreactive. d) peak (3) identified as LL-37 (MW 4493). Data from single experiment representative of 3.

Figure 5.

**Processing of LL-37 enhances antimicrobial activity**

The antimicrobial activity of cathelicidin peptides was evaluated by radial diffusion assay against (a) *S.aureus*, (b) *E.coli*, (c) solution assay against *C.albicans* and (d) Group A Streptococci. The antimicrobial activity of each peptide was evaluated in 10%TSB/10mM phosphate buffer with several NaCl concentrations against *E.coli* (e) and *S.aureus* (f). Data shown are representative of triplicate determinations.

**Figure 6.****Processing of LL-37 decreases ability to stimulate keratinocyte IL-8**

Cathelicidin peptides were added to culture of normal human keratinocytes at a final concentration of 3 or 10  $\mu$ M then IL-8 release determined after 8 hr. All samples were endotoxin free by limulus assay.

Data are mean  $\pm$ SEM of triplicate determinations.

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FIGURE 1

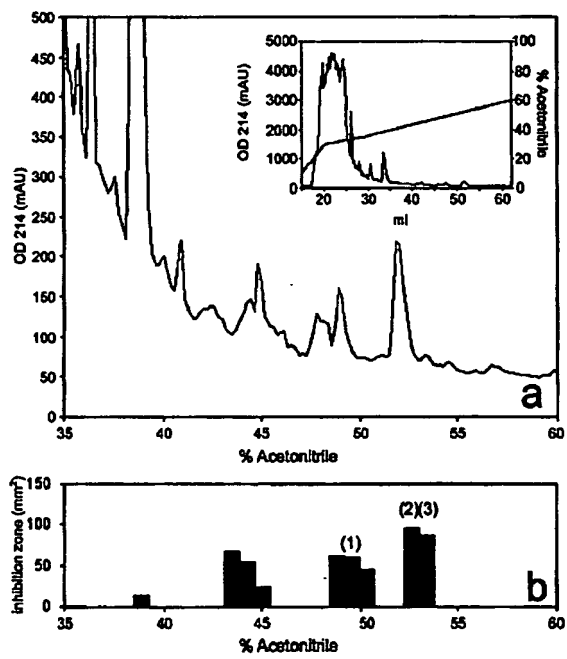




FIGURE 2

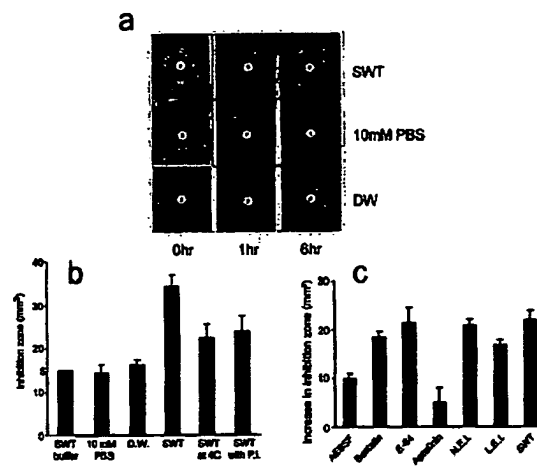


FIGURE 3

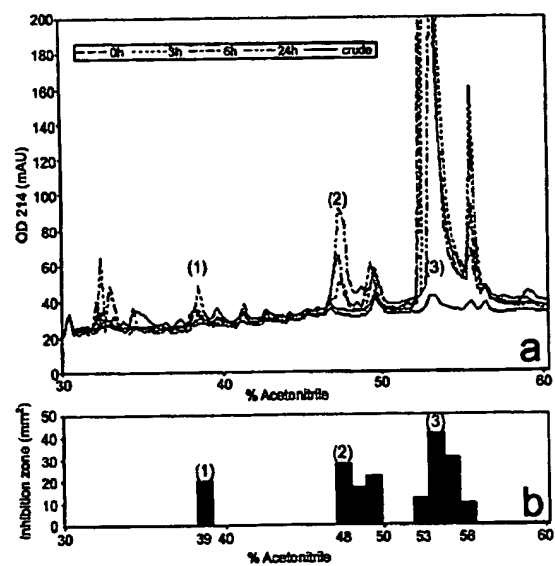


FIGURE 4

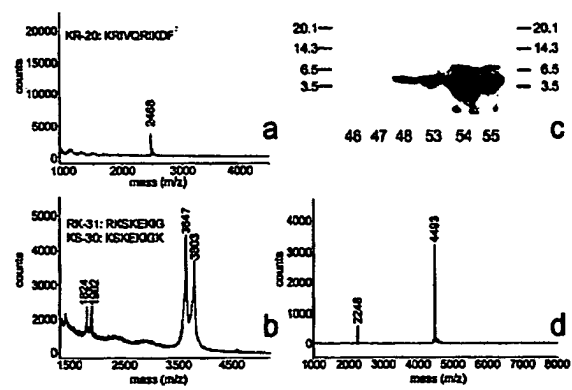


FIGURE 5

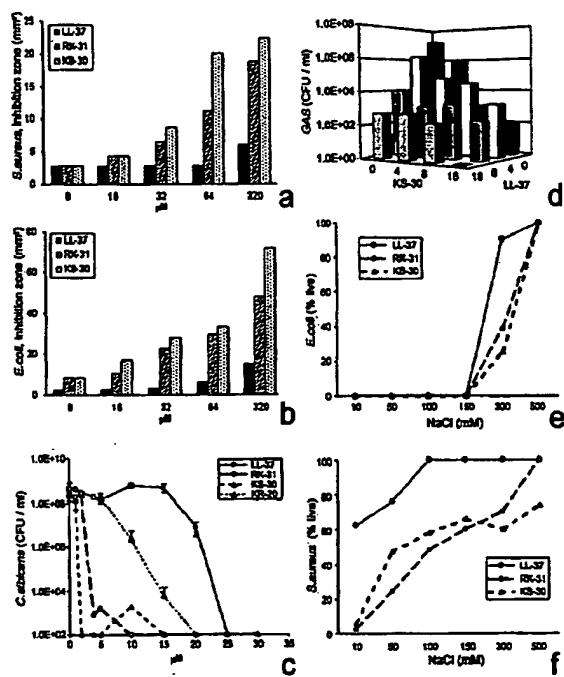


FIGURE 6

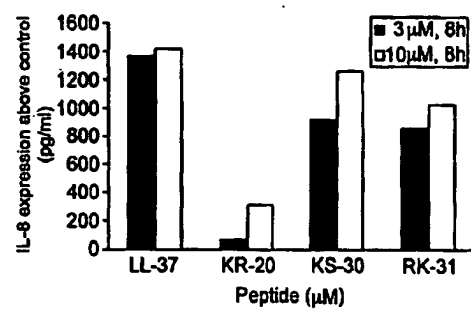


FIGURE 7

% Inhibition of Staph Aureus by LL-37 in 100mM NaCl, 10% FCS -  
2hrs to Overnight

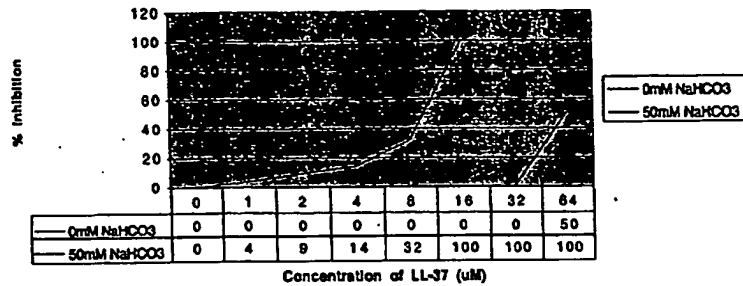


FIGURE 8

Growth of Staph Aureus in 100mM NaCl, 10% FCS Media Containing Various NaHCO3 Concentrations

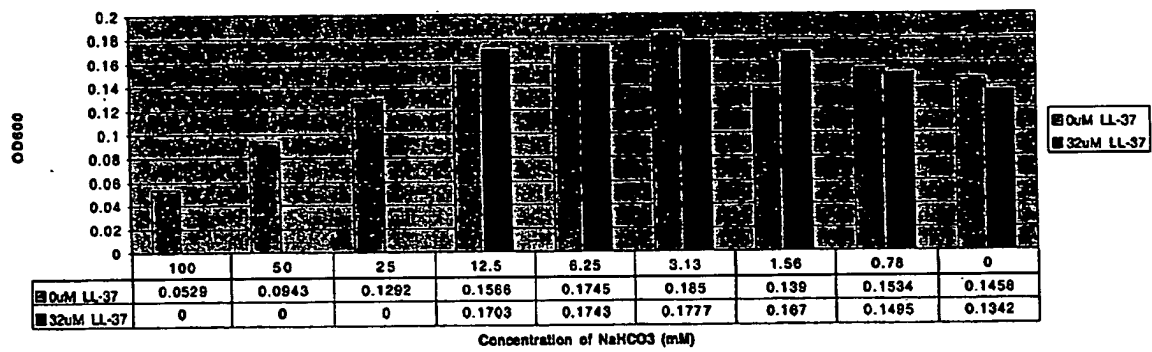
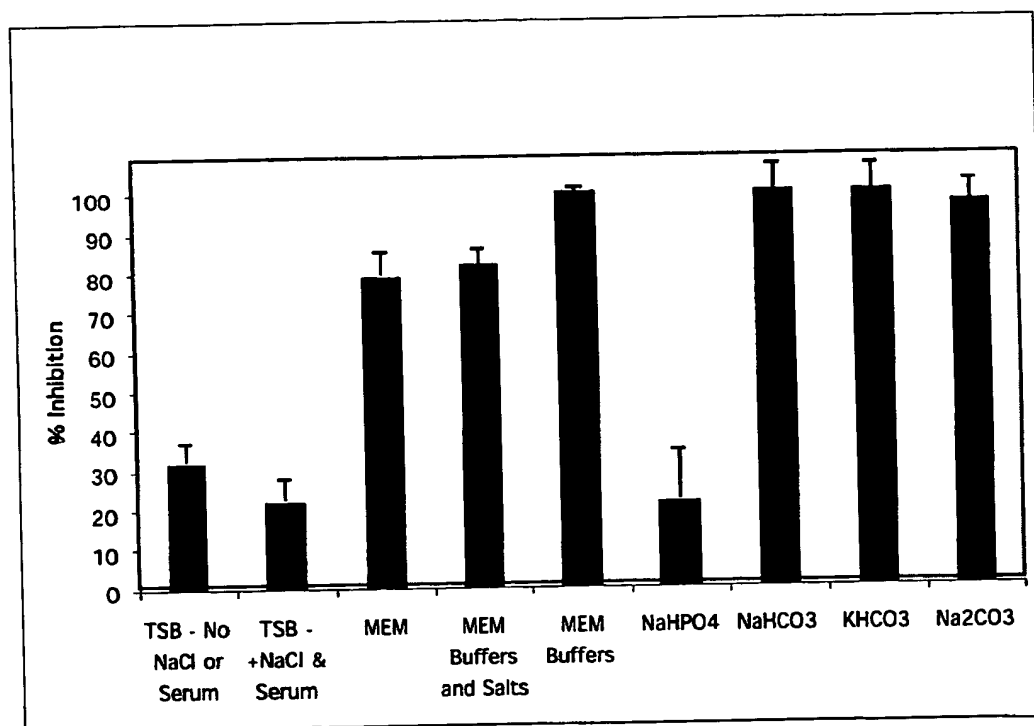
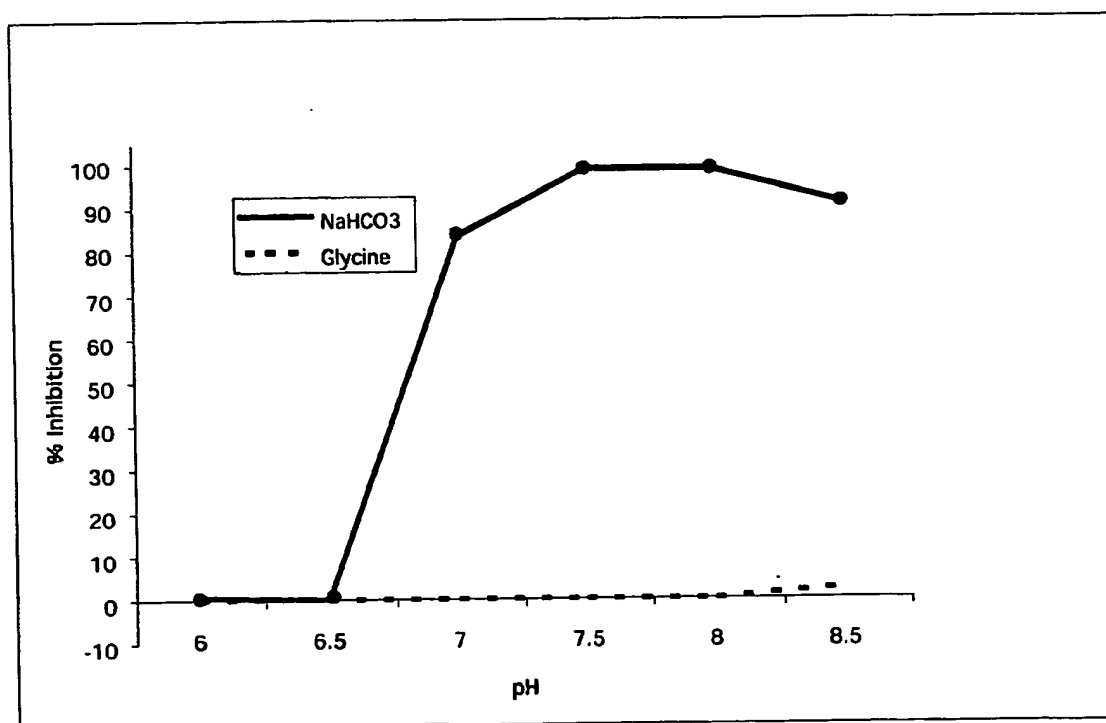


FIGURE 9



Staph aureus  
 32  $\mu$ M LL-37  
 20% TSB  
 10% FCS  
 pH 7.4

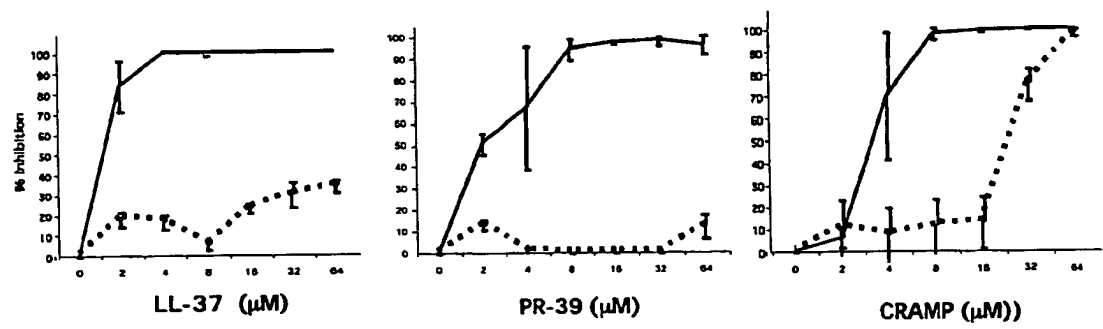
FIGURE 10



Staph aureus  
32 uM LL-37  
20% TSB  
150 mM NaCl  
10% FCS



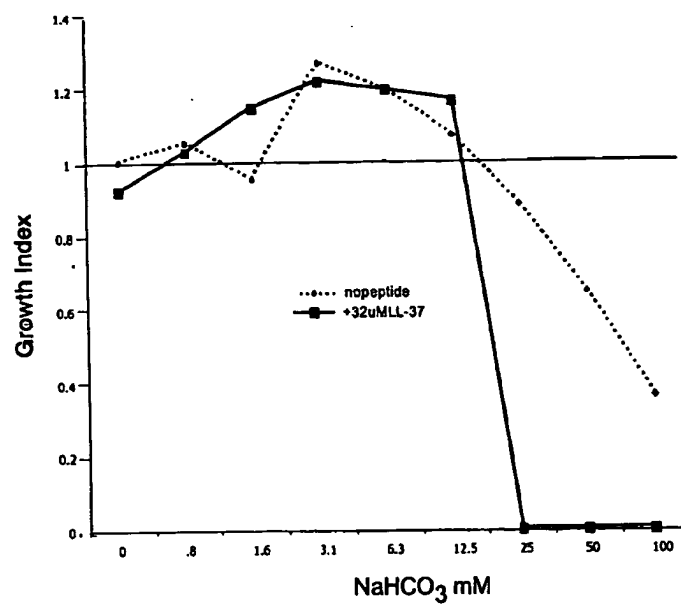
FIGURE 11



*Staph aureus*  
20% TSB  
NO NaCl  
No FCS

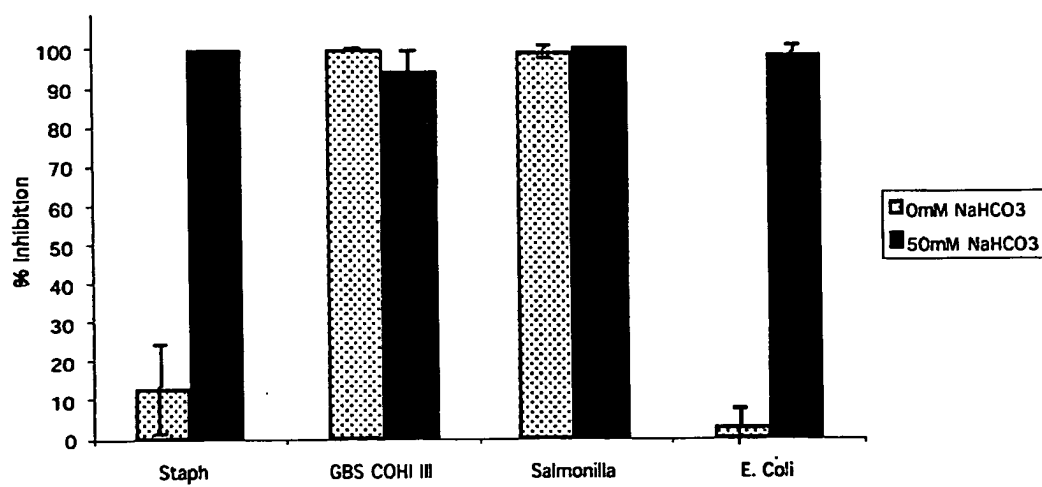
--- 0mM NaHCO<sub>3</sub>  
— 50mM NaHCO<sub>3</sub>

FIGURE 12



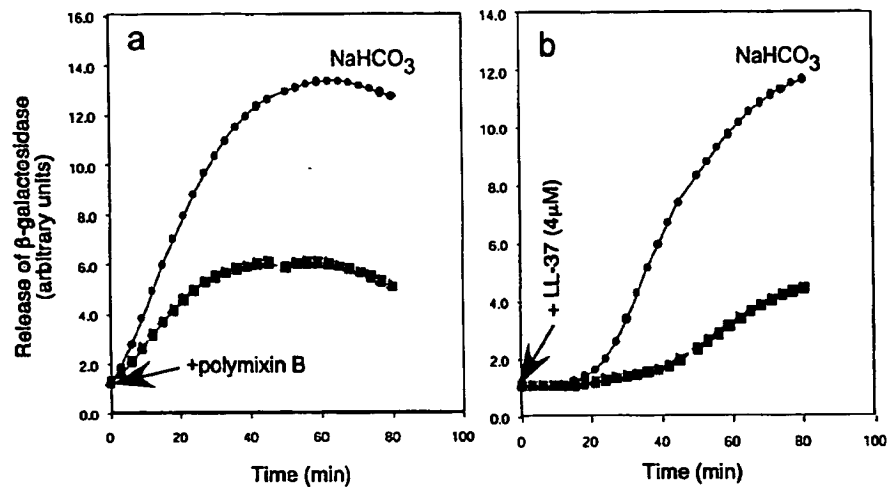
Staph aureus  
pH 7.4  
20% TSB  
150 mM NaCl  
10% FCS

FIGURE 13



Cramp at 16 uM  
20% TSB  
no NaCl or FCS  
pH 7.4

FIGURE 14



e. coli inner membrane permeability  
 no NaCL, FCS  
 pH 7.4  
 data are OD 420 with antibiotic/no antibiotic

FIGURE 15

Effect of EF on the Inhibitory Activity of Various Antimicrobial Peptides on Staph Aureus  
(+150mM NaCl and 10% FCS)

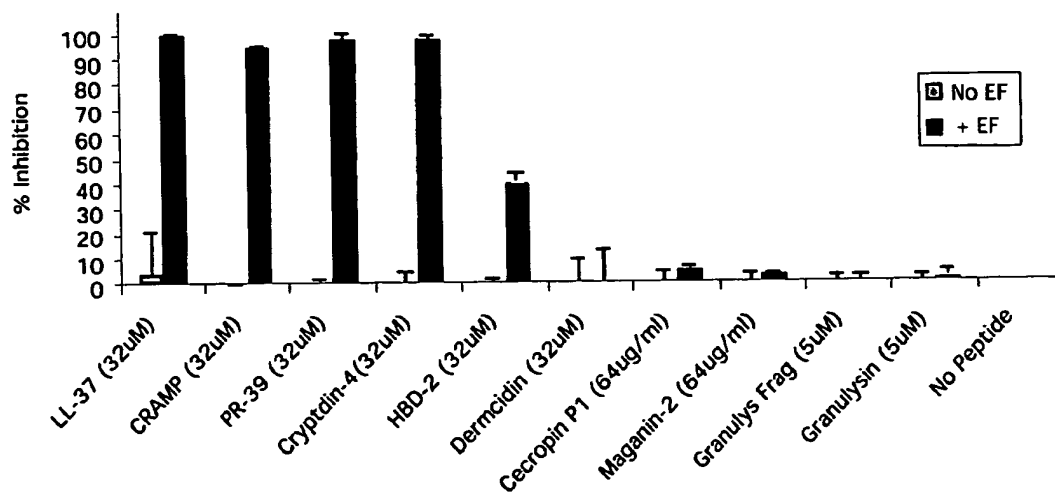


FIGURE 16

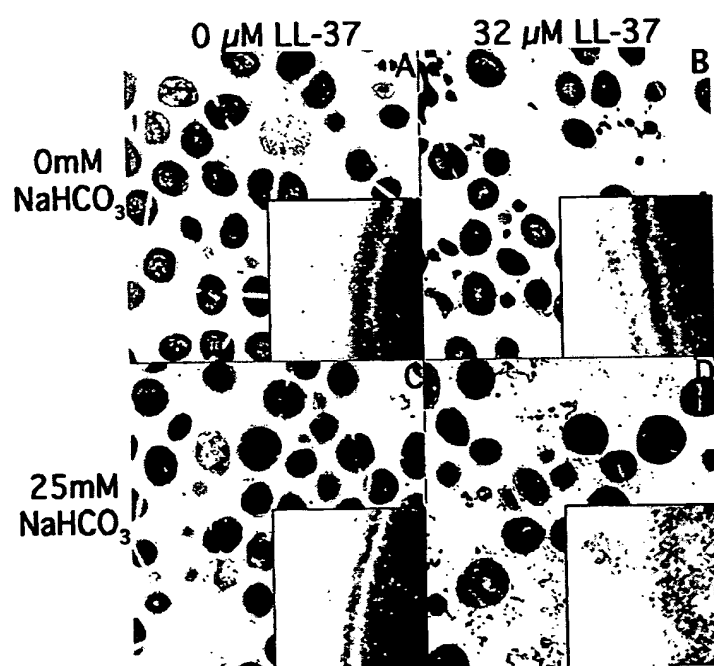


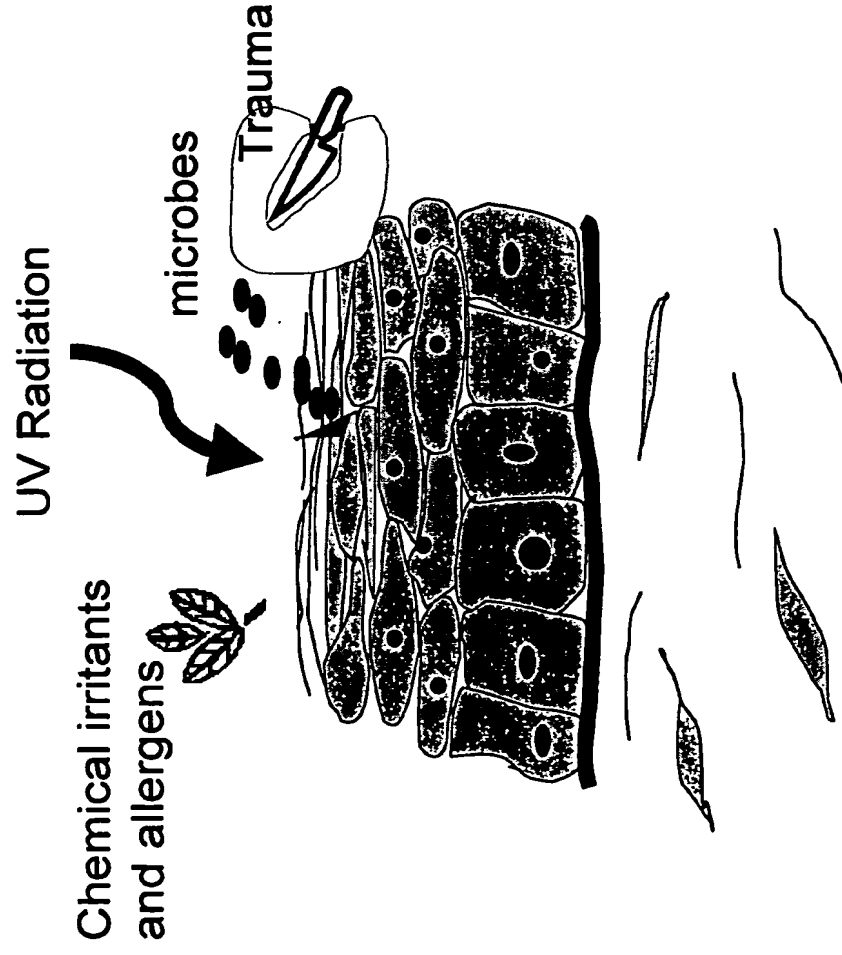


FIGURE 17 - 40pg

# Antimicrobial Peptides and Innate defense of Skin

Richard L. Gallo, M.D., Ph.D.  
University of California, San Diego

## Skin as a simple Barrier

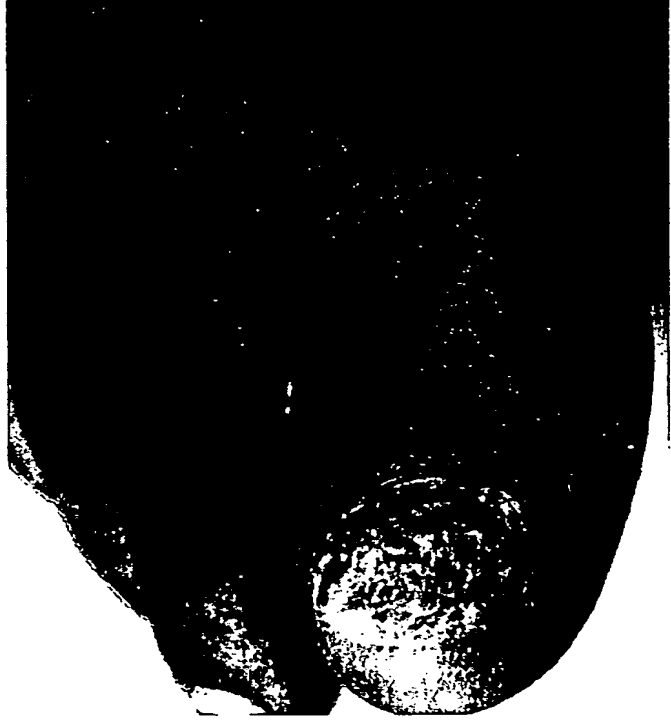


## Immune Defense System

1. Physical Barrier
2. Chemical (NO, H<sub>2</sub>O<sub>2</sub>)
3. Inflammatory
  - Cytokines
  - Chemokines
  - Neuropeptides
  - Eicosanoids
4. Cellular
  - Neutrophils
  - Macrophages
  - Natural Killer
5. Adaptive
  - Dendritic cells
  - T and B cells



How do we explain common skin diseases with this model?



Onychomycosis:

Nail Plate infected

No opportunity for direct cell-mediated defense

How do we explain common skin diseases with this model?



**Psoriasis:**

Triggered by:  
stress, trauma, infection

Patients have fewer infections

How do we explain common skin diseases with this model?



Atopic Dermatitis, Eczema Herpeticum:

Increased inflammation  
but  
Increased infections

How do we explain common skin diseases with this model?



Rosacea:

Triggered by:  
vasodilation, bacteria

Patients have chronic infection  
without apparent immune deficit

The answer may be to understand “innate immunity”

## Innate Immunity

- non-adaptive (no need to learn)
- non-clonal (not dependent on cell proliferation)
- rapid response to danger (injury or infection)
- evolutionarily conserved (insects, plants)
- adapted by mammals (acts on cellular immunity)

Insects and lower organisms depend on innate immunity

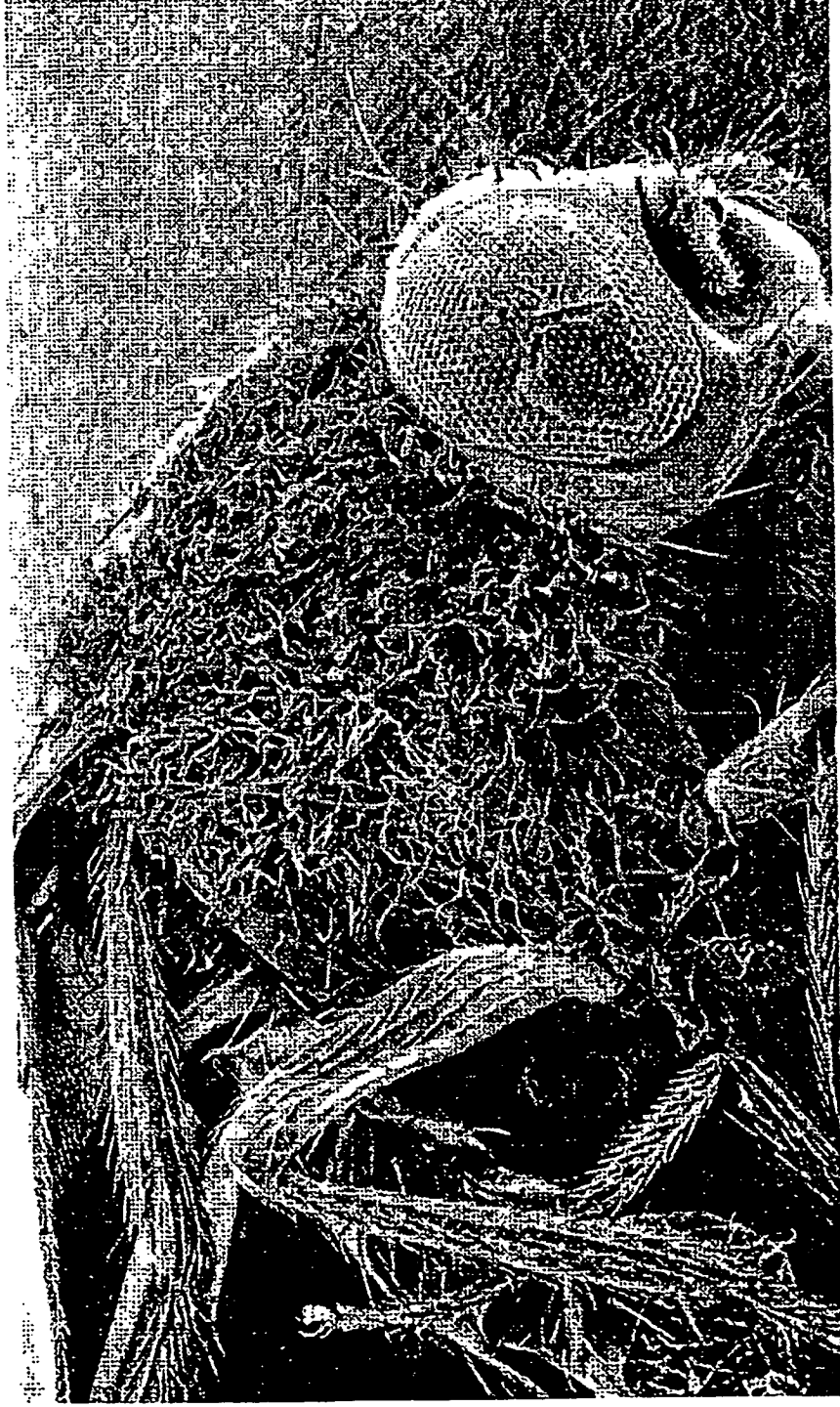


Figure 5. Germinating Hyphae of *A. fumigatus* on a Dead *Drosophila*  
Scanning electron micrograph of a *Drosophila* adult that succumbed  
to infection by *A. fumigatus* and is covered with germinating hyphae  
(200 $\times$  magnification).

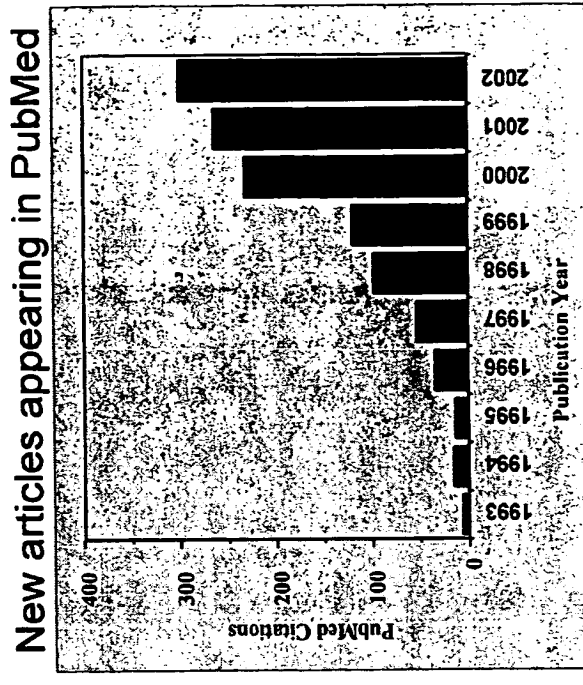
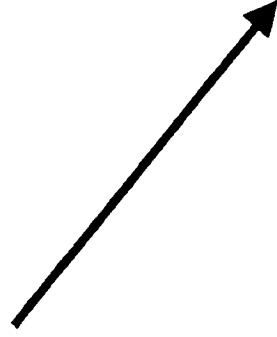
# Innate Immunity in 2003

Found in all multicellular organisms

- Dependent on specific pattern recognition
- Multiple effector mechanisms

Close interactions with adaptive immunity

- Increasing interest



Essential element in skin defense



## Multiple Effector Elements of Innate Immunity

### Type

Inorganic molecules

Simple Organics

Binding Proteins

Cytokines

Cells

Proteins

### Examples

HCl, H<sub>2</sub>O<sub>2</sub>, nitric oxide

fatty acids, lipids

Mannose binding protein

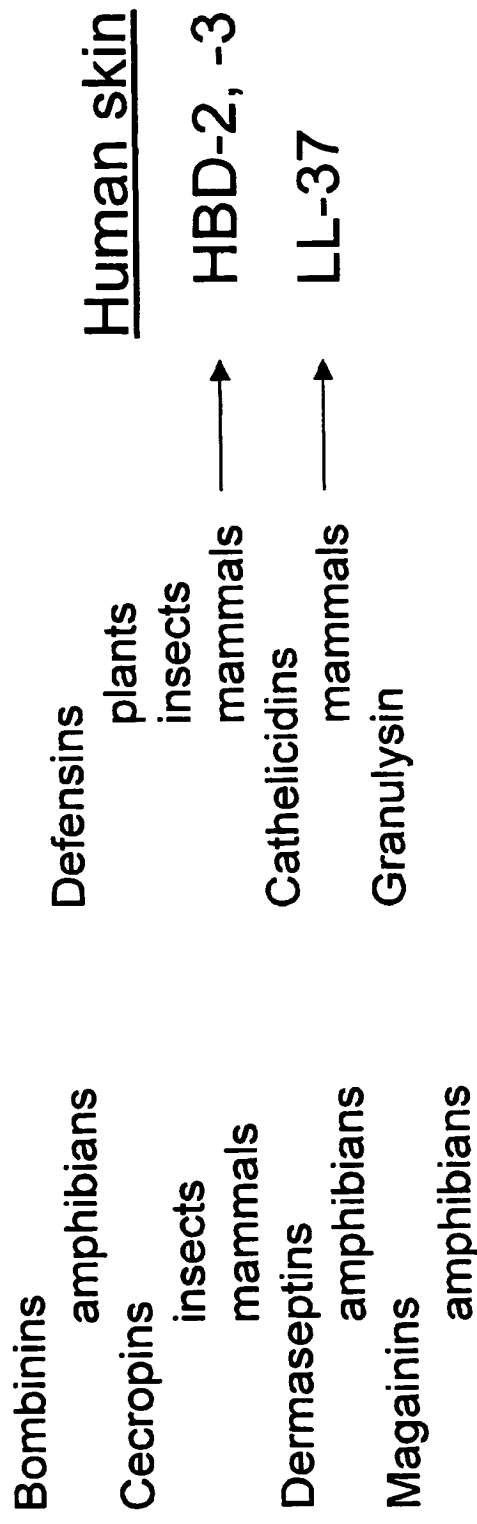
IL-10, IL-12, TNF-alpha

Macs, Polys, NK

BPI, lysozyme,

## Antimicrobial Peptides

## Many Peptides with Antimicrobial Action



**Over 800+ peptides known**

## In Skin, Injury or Infection Triggers Antimicrobial Peptide Expression

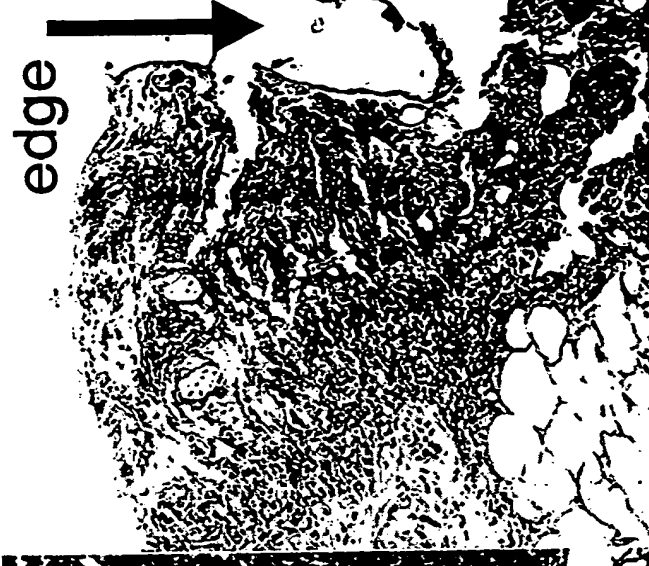
Normal skin



Strep infection



12 hr Wound

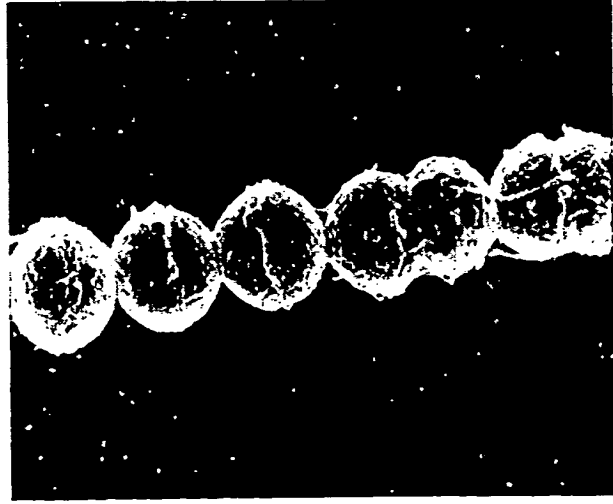
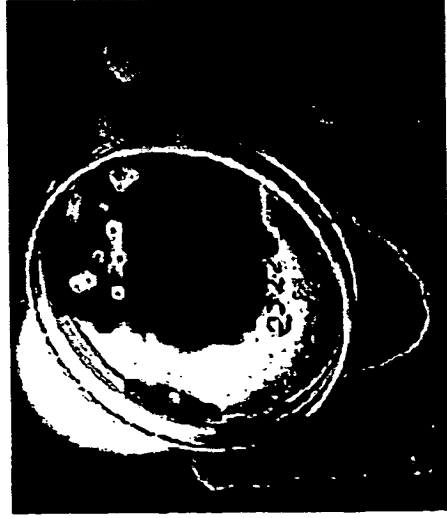


Brown staining= cathelicidin peptide immunoreactivity

Is an increase in antimicrobial peptides  
important?

# GROUP A STREPTOCOCCUS (*S. PYOGENES*)

---

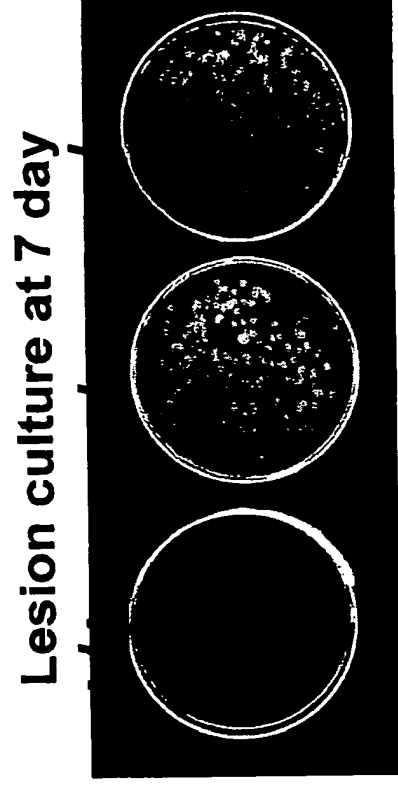
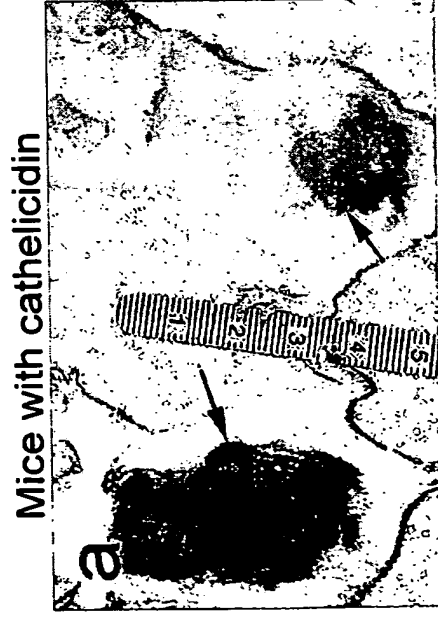


## SENSITIVE TO CATHELICIDINS

CRAMP: MIC = 2 - 8  $\mu$ M

LL-37: MIC = 8 - 24  $\mu$ M

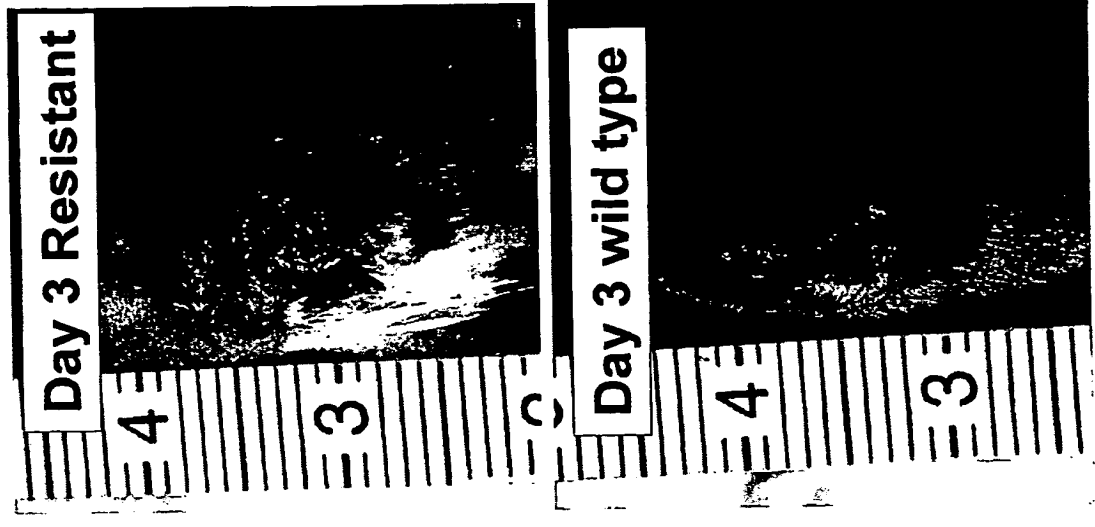
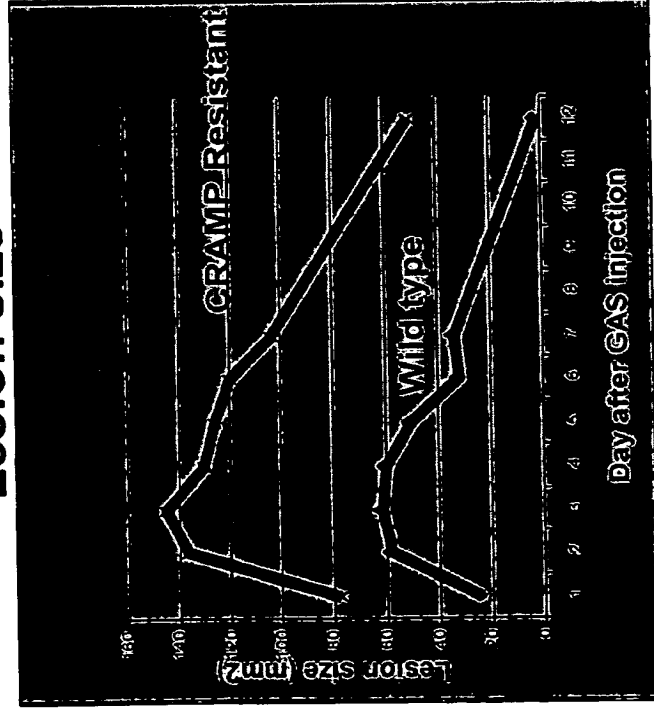
In mice, cathelicidin knock-outs are more susceptible to  
Invasive Group A *Streptococcus*



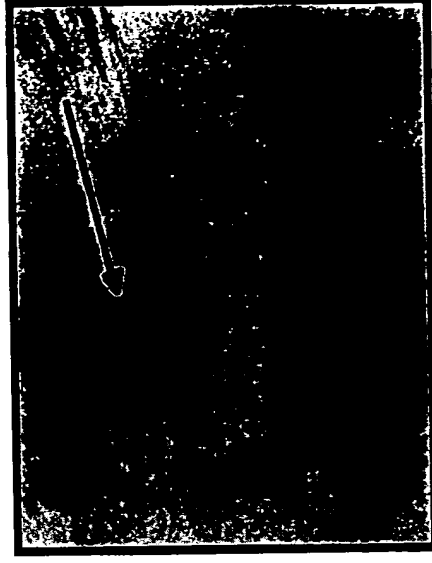
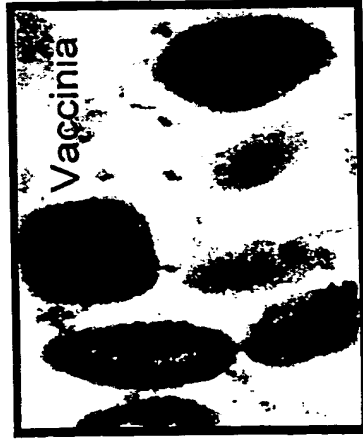
**day 7**

Bacteria (GAS) that are cathelicidin resistant are more pathogenic

### Lesion size



Cathelicidins also provide defense against viral infection



NO LL-37

LL-37 5  $\mu$ M

NO LESION 15/16 CONTROL MICE

LL-37 ( $\mu$ M)	Altered virions/ virion number (%)	
0	1/23	(4%)
5	19/28	(67%)
25	27/30	(90%)



VESICULAR LESION 4/6 CRAMP --/--



## **Conclusion:**

**The presence of antimicrobial peptides is essential for normal defense against certain bacterial and viral skin infections**

How are the cathelicidin antimicrobial peptides used by the skin?

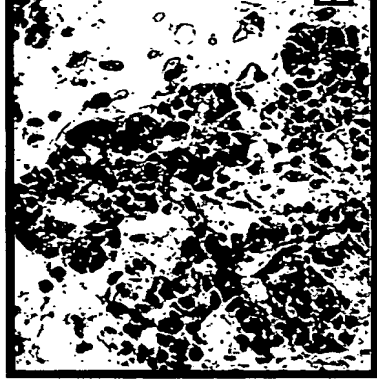
Cathelcidins are expressed at the interface



**SKIN**



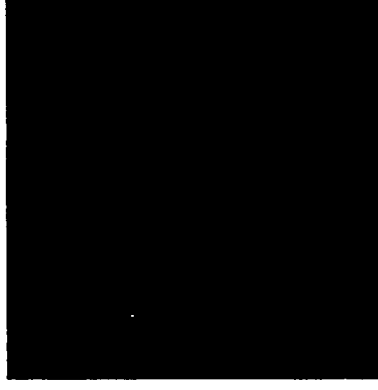
**COLON**



**SALIVARY GLAND**



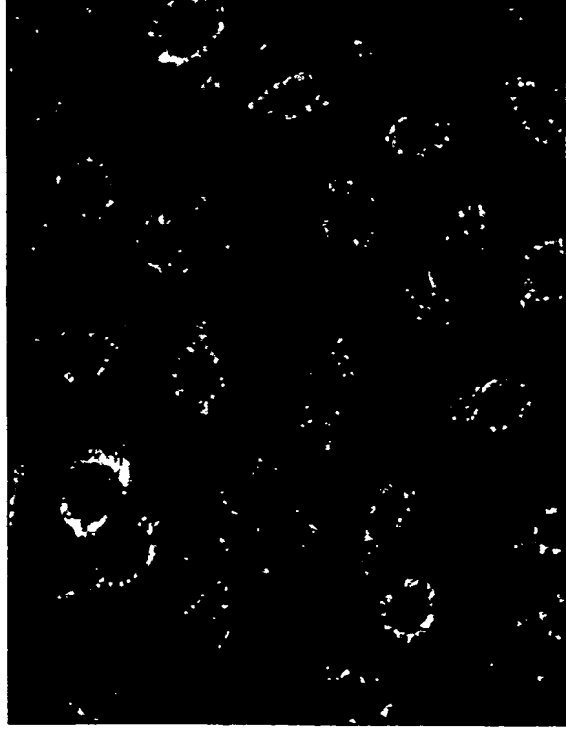
**NEUTROPHIL**



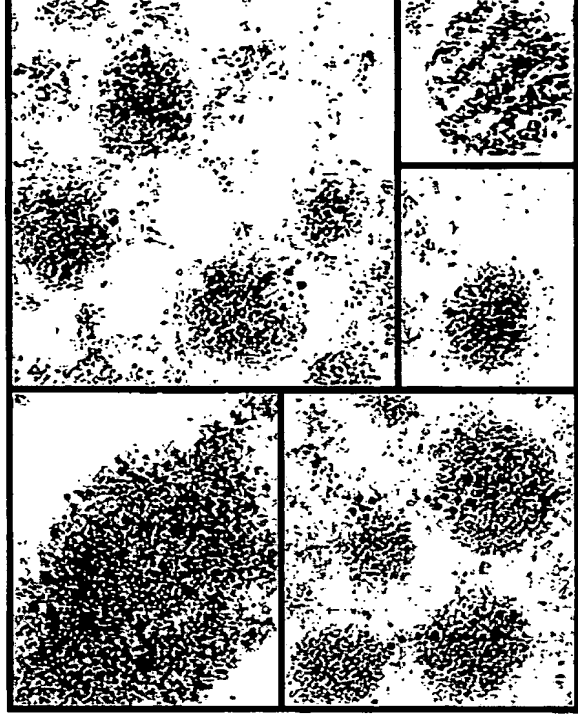
**MAST CELL**

# CATHS ARE STORED IN KERATINOCTYE GRANULES

FITC Anti-LL37



Anti-LL37 immuno gold EM



Caths are expressed in the nail apparatus

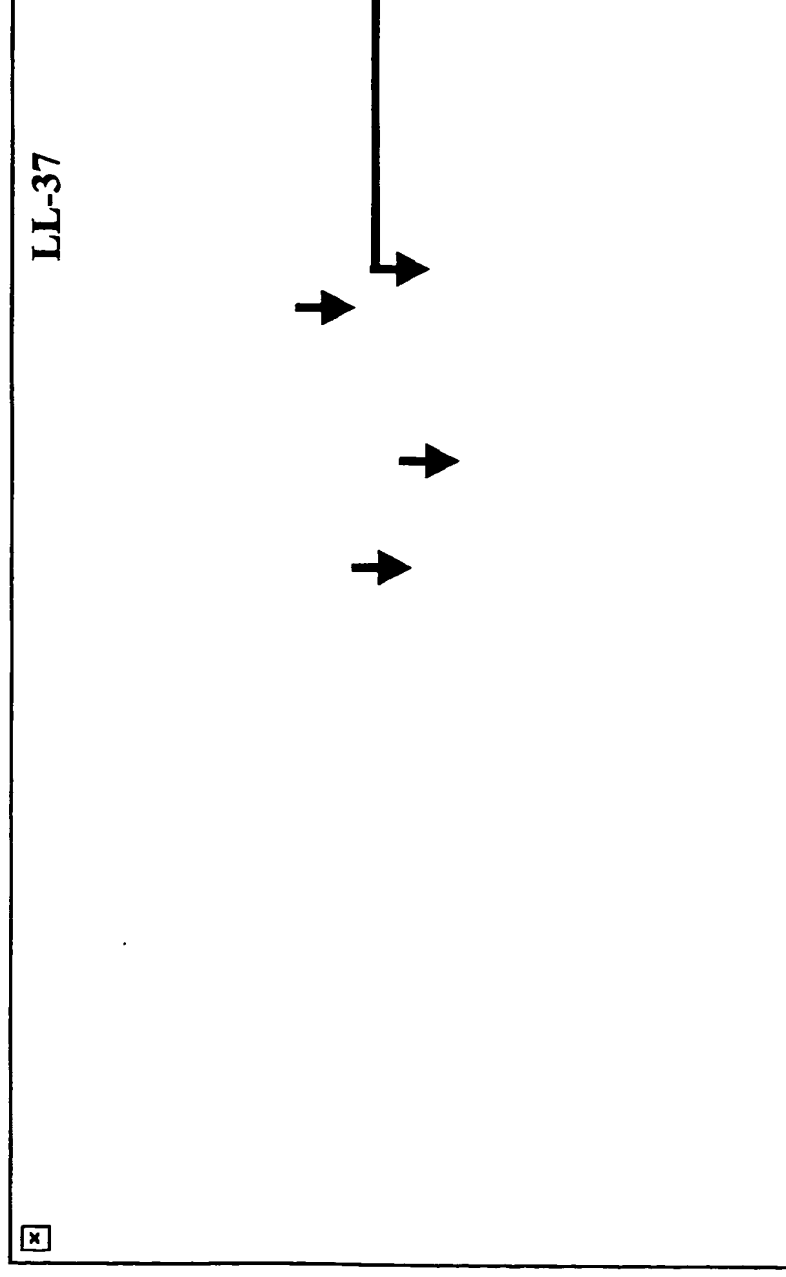


Is this an  
answer to  
normal nail  
defense?

## Cathelicidin peptides discovered in sweat with increased activity



Eccrine gland and duct  
stain for cathelicidin



Work of Dr. M. Murakami, now a Dermatology resident at Asahikawa

Does this correlate with human disease?

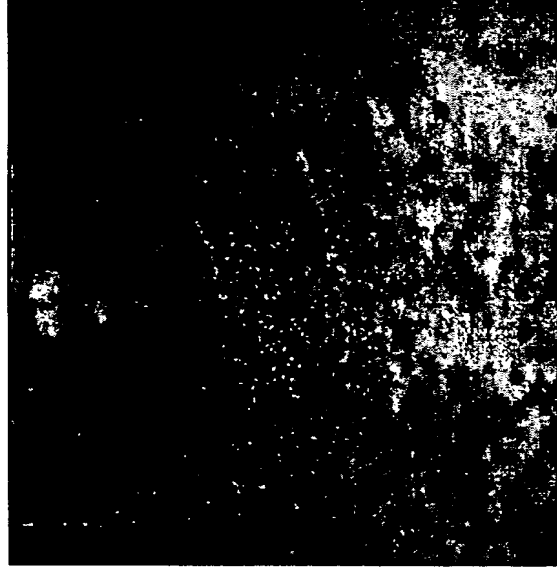
Atopic patients are susceptible to infection and have inflammation.  
What is their expression of antimicrobial peptides?

Impetigo



Staph  
Strep

Eczema Herpeticum



HSV, VZV

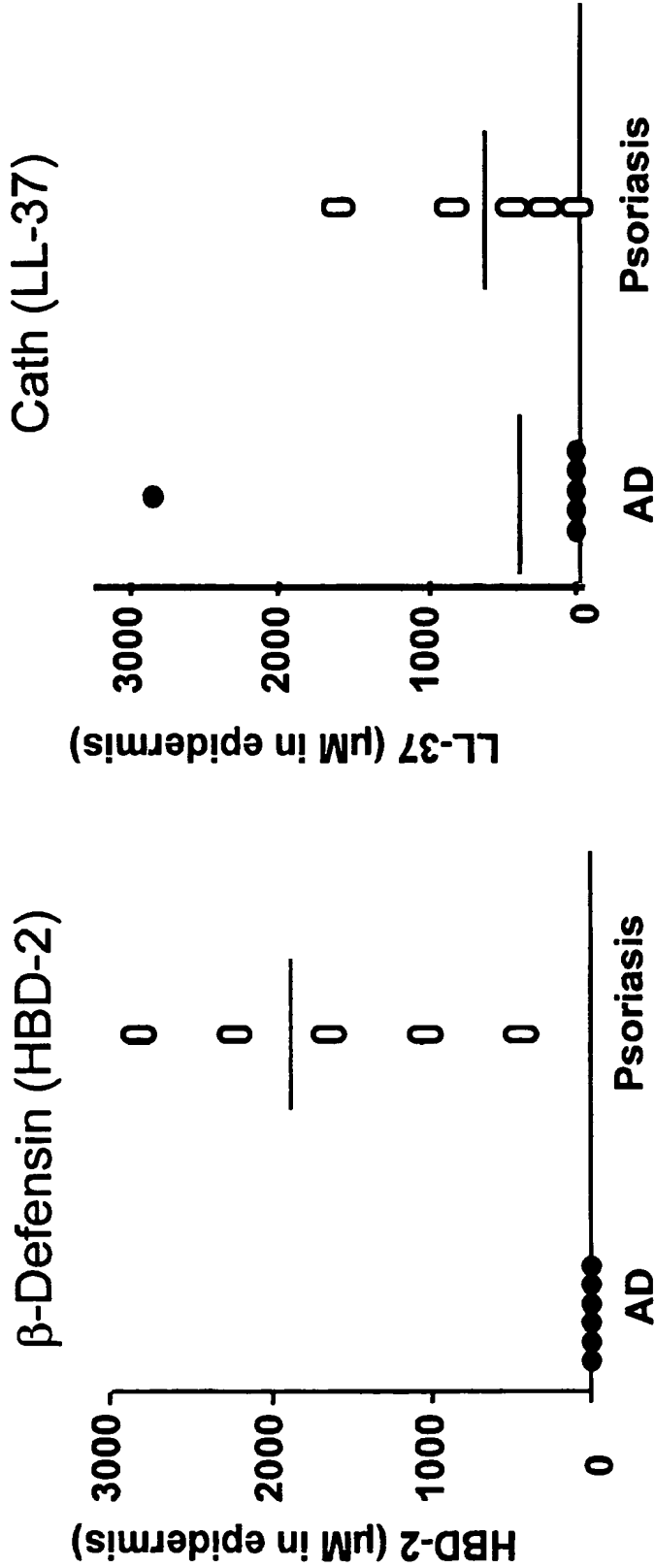
Eczema Vaccinatum



Cow pox



Compared to psoriasis, atopic patients do not respond to inflammation with a increase in antimicrobial peptides



Ong et al. (2002) N Engl J Med 347:1151-60

Work of Dr. T. Ohtake, now faculty in Medicine at Asahikawa

## Conclusions

Cathelicidins are produced by several cells in the skin.  
A lack of normal production correlates with disease

Psoriasis

Overproduction ▲ Protection



Atopic Dermatitis

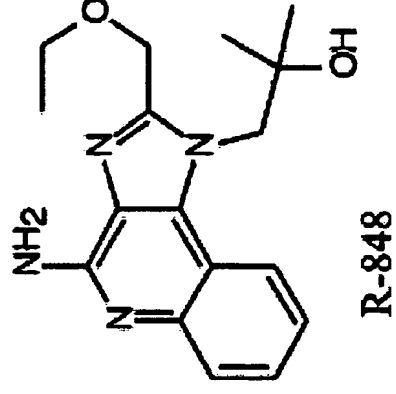
Underproduction ▼ Infection



Can our increased understanding of innate immunity  
and antimicrobial peptides be used for therapy?

CC(C)CN1C=NC2=C(N)N=CN=C2C3=CC=CC=C13

**Imiquimod**



**Effective for:**

Common warts, condylomata, molluscum, BCC, Bowens, Bowenid papulosis, AK, lentigo maligna, stucco keratosis, Porokeratosis of Mibelli

## Antimicrobial peptides have been successfully used in preliminary animal models

Protection of burn wounds against infection  
(2001) Crit Care Med 29:1431

Skin wounds  
(2001) Blood 97:297

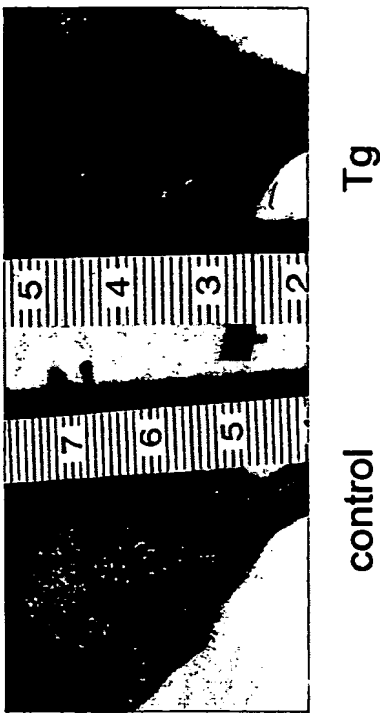
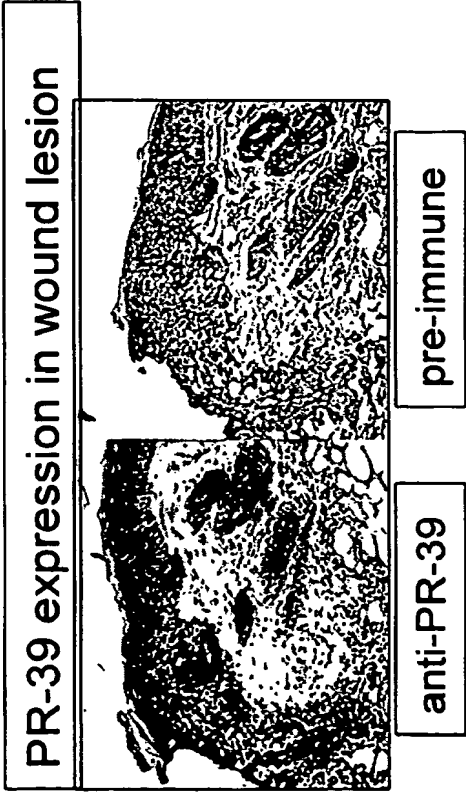
Peritonitis  
(1997) Antimicrob Agents Chemother 41:1738

Cardiac angiogenesis  
(2000) Nat Med 6:49

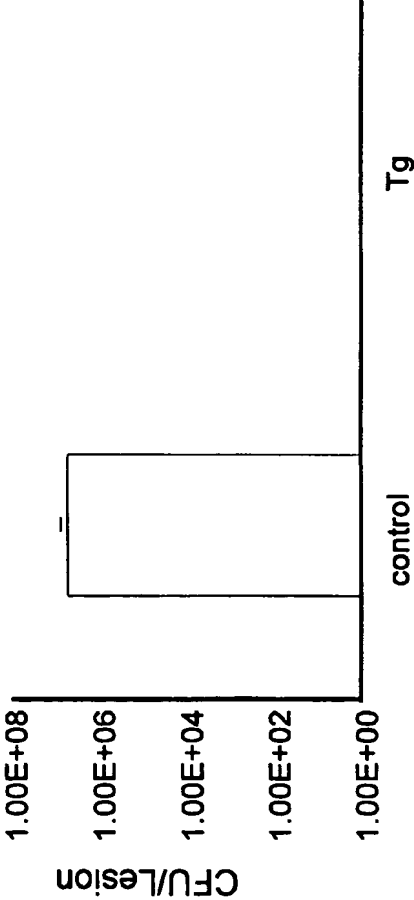
Endotoxic Shock  
(1998) Infect Immun 66:1861

Lung Infection  
(1998) J Clin Invest 102:874

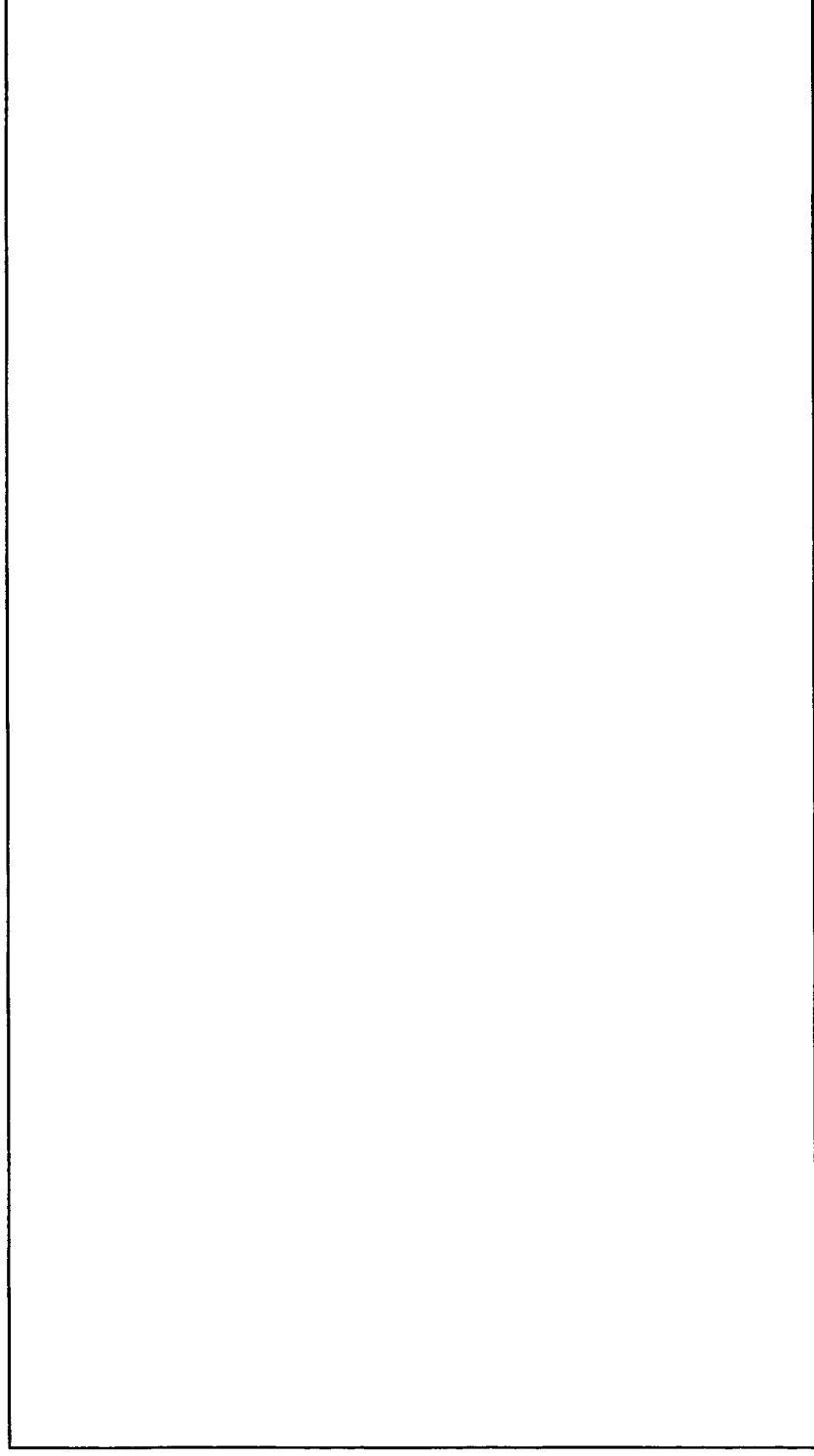
Antimicrobial gene therapy is effective in mice



Live bacteria in lesion (day6)



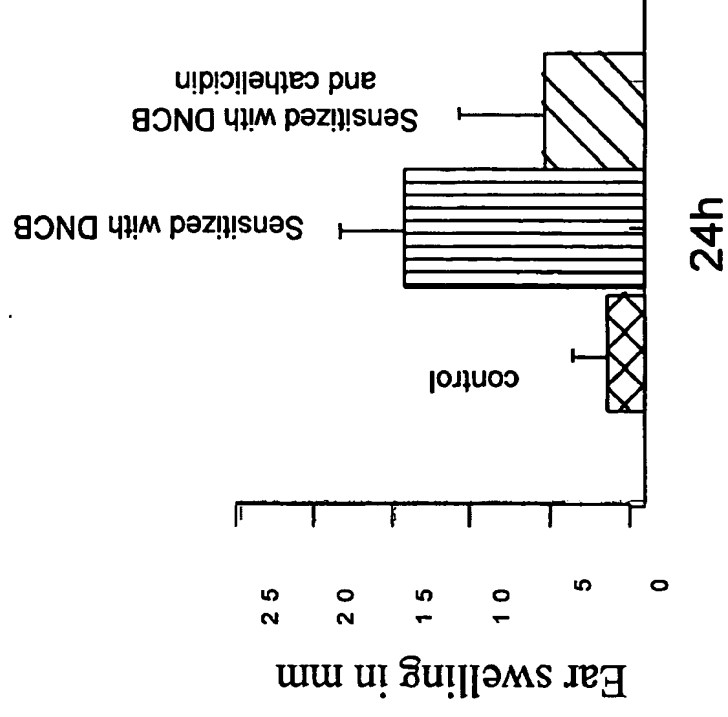
Human trials are underway



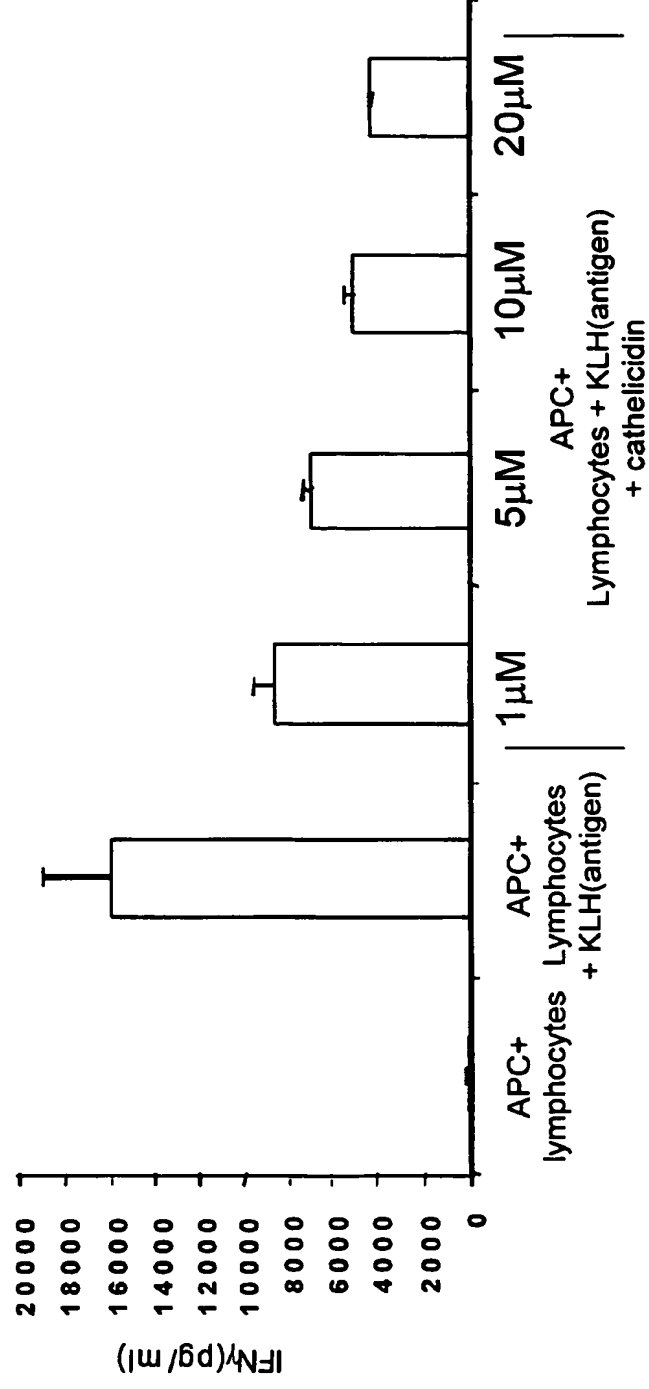
However,  
Antimicrobial Peptides do more than kill microbes!



## Cathelicidin blocks allergic contact dermatitis in mice



## Cathelicidin blocks antigen presentation



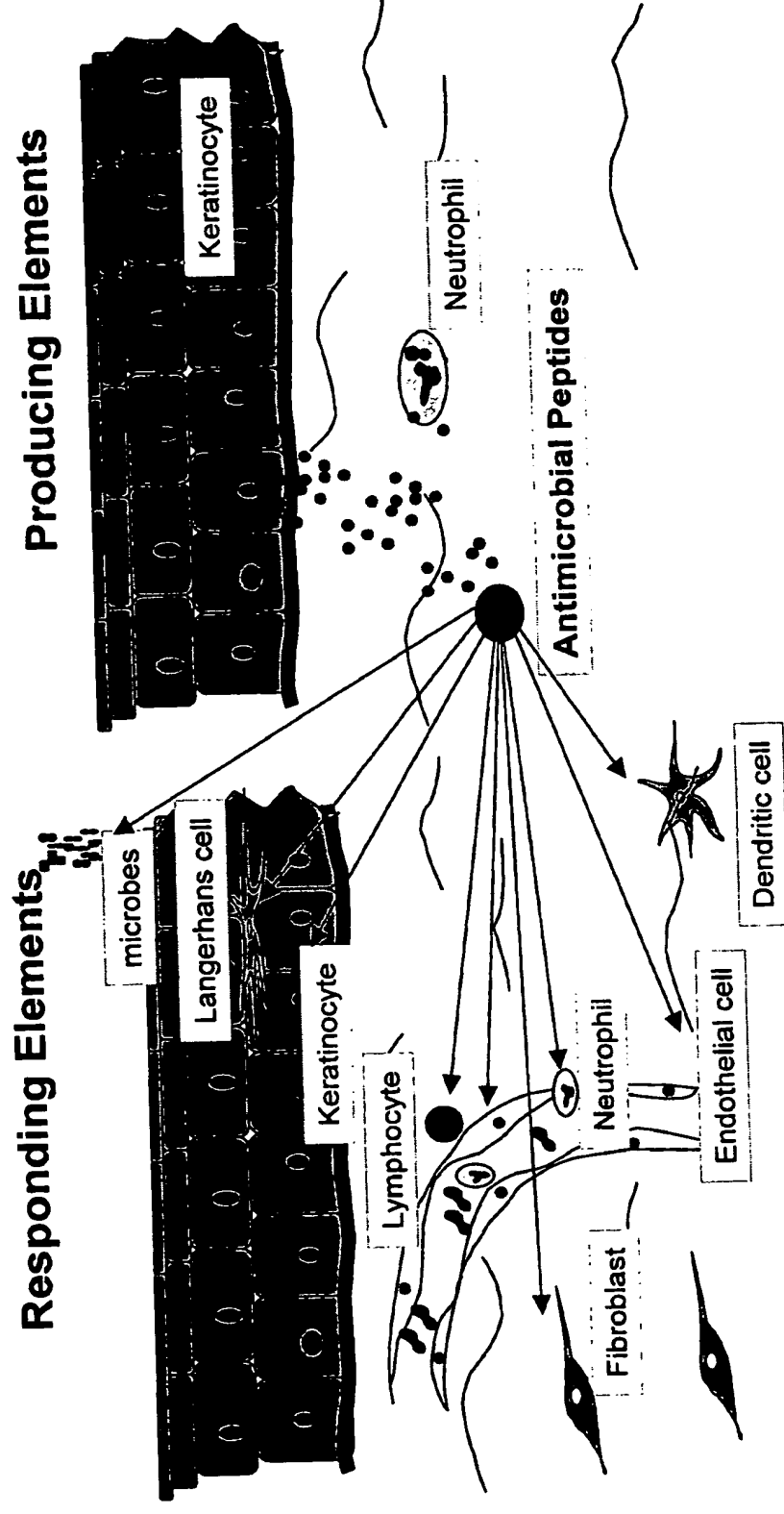
But chronic administration of cathelicidin is proinflammatory



Figure 5

- Antimicrobial peptides are important to innate immune defense
- Defects in innate immunity may explain certain skin diseases
- Action of antimicrobial peptides are part of the whole immune system

# SUMMARY: Antimicrobial Peptides in the Skin



Gallo Lab

Anna Di Nardo  
Robert Dorschner  
Xavier Lauth  
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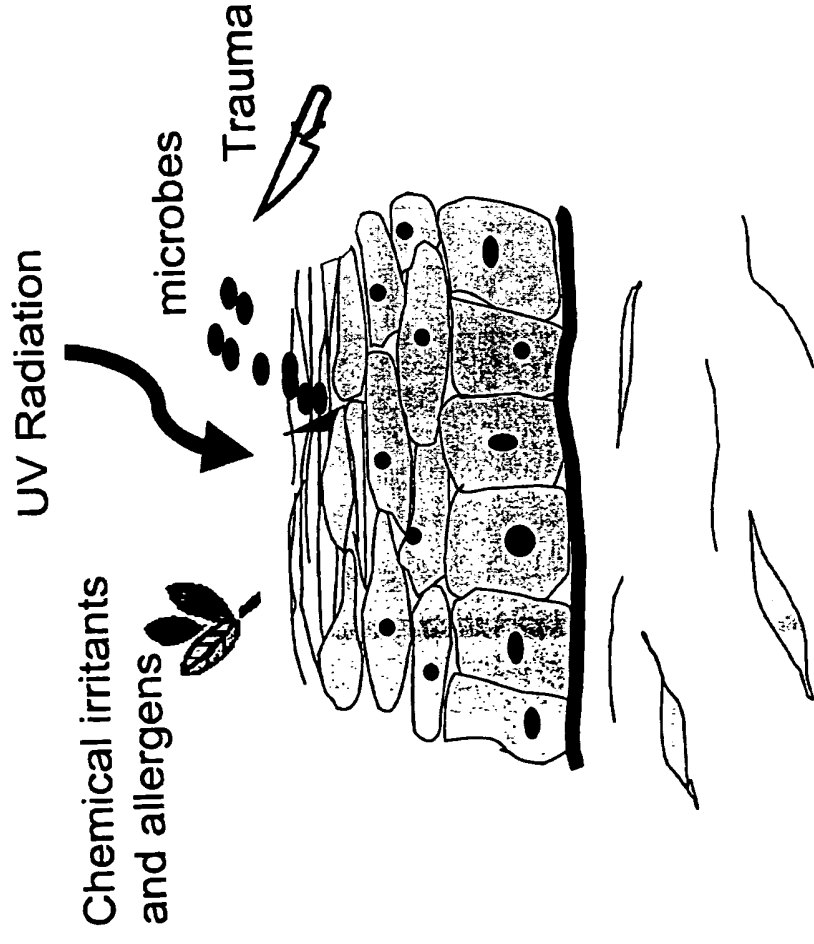


FIGURE 18-40 pg 3

# Antimicrobial Peptides and Innate defense of Skin

Richard L. Gallo, M.D., Ph.D.  
University of California, San Diego

## Skin as a simple Barrier



## Immune Defense System

- Physical Barrier
  - Chemical (NO, H<sub>2</sub>O<sub>2</sub>)
  - Inflammatory
    - Cytokines
    - Chemokines
    - Neuropeptides
    - Eicosanoids
  - Cellular
    - Neutrophils
    - Macrophages
    - Natural Killer
5. Adaptive
- Dendritic cells
  - T and B cells



How do we explain common skin diseases with this model?



Onychomycosis:

Nail Plate infected

No opportunity for direct cell-mediated defense

How do we explain common skin diseases with this model?

Psoriasis:

Triggered by:  
stress, trauma, infection

Patients have fewer infections

How do we explain common skin diseases with this model?



Atopic Dermatitis, Eczema Herpeticum:

Increased inflammation  
but  
Increased infections

How do we explain common skin diseases with this model?

Rosacea:



Triggered by:  
vasodilation, bacteria

Patients have chronic infection  
without apparent immune deficit

The answer may be to understand “innate immunity”

## Innate Immunity

- non-adaptive (no need to learn)
- non-clonal (not dependent on cell proliferation)
- rapid response to danger (injury or infection)
- evolutionarily conserved (insects, plants)
- adapted by mammals (acts on cellular immunity)

Insects and lower organisms depend on innate immunity

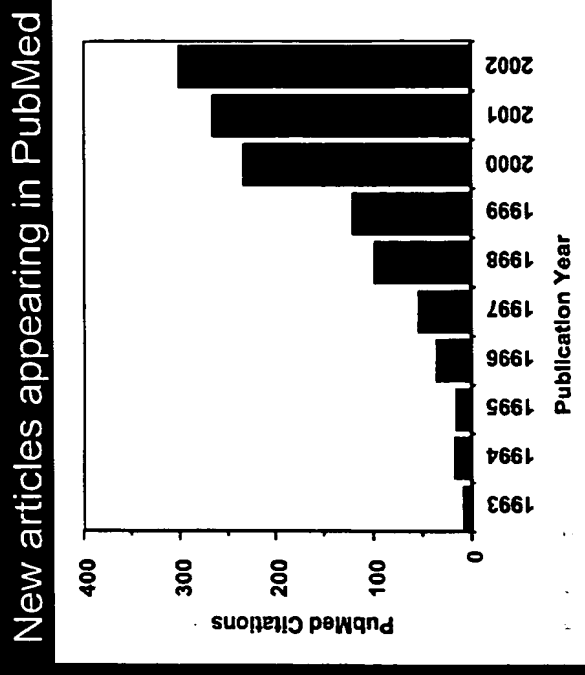


Figure 5. Germinating Hyphae of *A. fumigatus* on a Dead *Drosophila* Scanning electron micrograph of a *Drosophila* adult that succumbed to infection by *A. fumigatus* and is covered with germinating hyphae (200× magnification).

## Innate Immunity in 2003

- Found in all multicellular organisms
- Dependent on specific pattern recognition
- Multiple effector mechanisms
- Close interactions with adaptive immunity
- Increasing interest

Essential element in skin defense





## Multiple Effector Elements of Innate Immunity

### Type

Inorganic molecules

Simple Organics

Binding Proteins

Cytokines

Cells

Proteins

### Examples

HCl, H<sub>2</sub>O<sub>2</sub>, nitric oxide

fatty acids, lipids

Mannose binding protein

IL-10, IL-12, TNF- $\alpha$

Macs, Polys, NK

BPI, lysozyme,

## Antimicrobial Peptides

## Many Peptides with Antimicrobial Action

Bombinins			
amphibians	Defensins		
Cecropins	plants		<u>Human skin</u>
insects	insects		
mammals	mammals	→	HBD-2, -3
Dermaseptins	Cathelicidins		
amphibians	mammals	→	LL-37
Magainins	Granulysin		
amphibians			

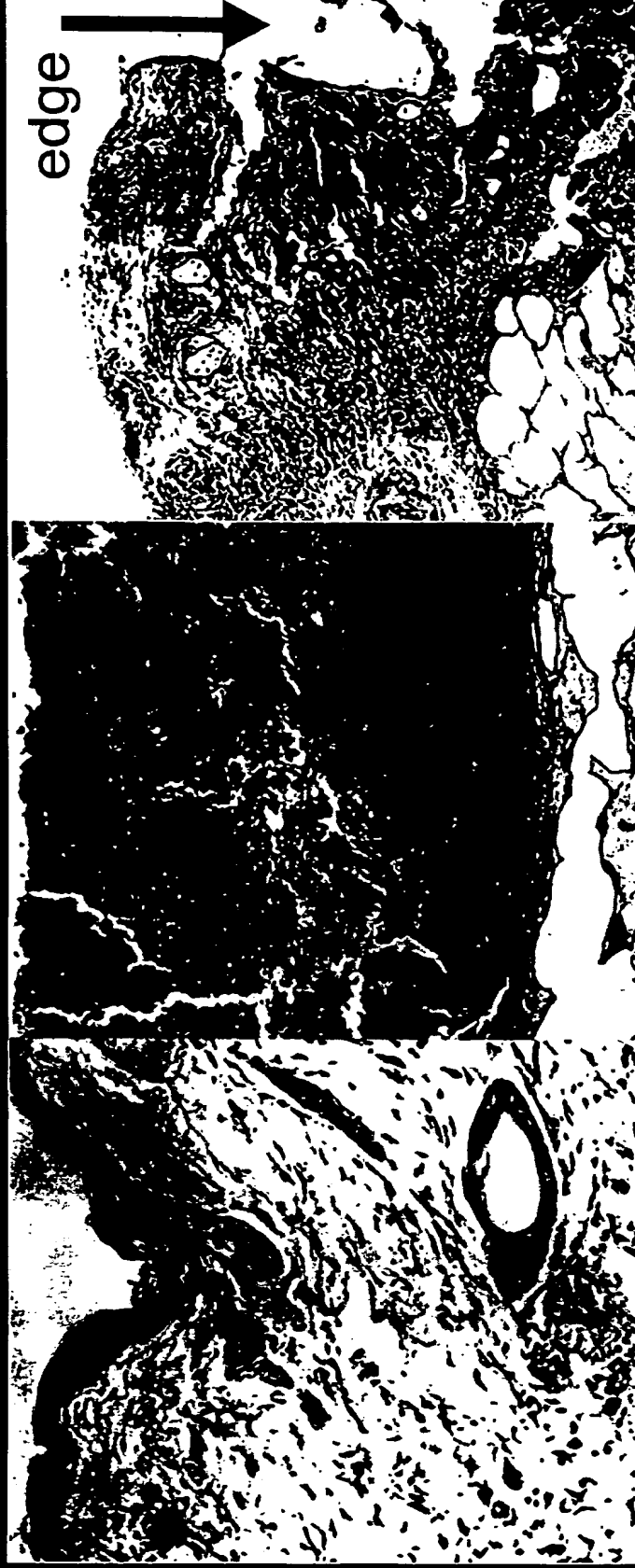
**Over 800+ peptides known**

## In Skin, Injury or Infection Triggers Antimicrobial Peptide Expression

Normal skin

Strep infection

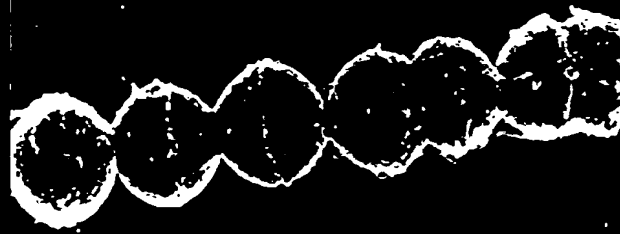
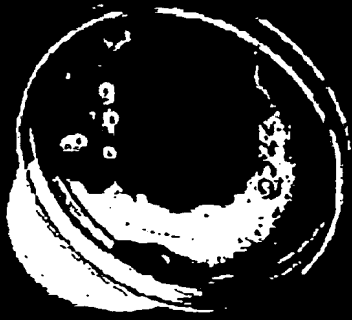
12 hr Wound



Brown staining= cathelicidin peptide immunoreactivity

Is an increase in antimicrobial peptides  
important?

# Group A Streptococcus (*S. pyogenes*)



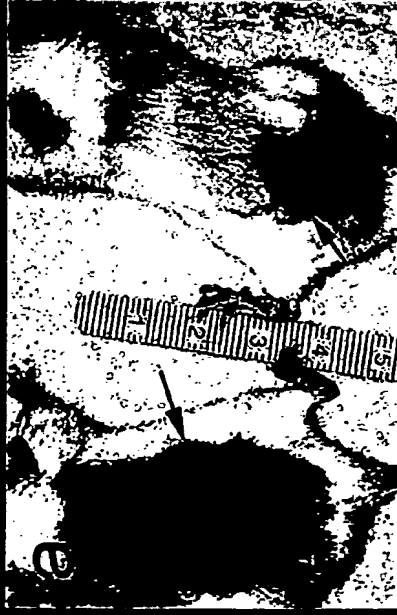
Sensitive to Cathelicidins

CRAMP: MIC = 2 - 8  $\mu$ M

LL-37: MIC = 8 - 24  $\mu$ M

# In mice, cathelicidin knock-outs are more susceptible to Invasive Group A *Streptococcus*

Mice with cathelicidin



Mice with no cathelicidin



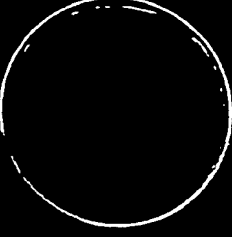
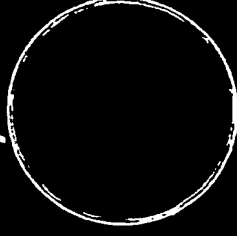
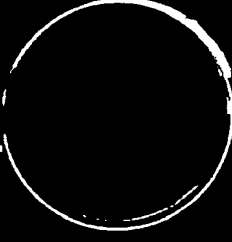
day 7

Lesion culture at 7 day

+/+

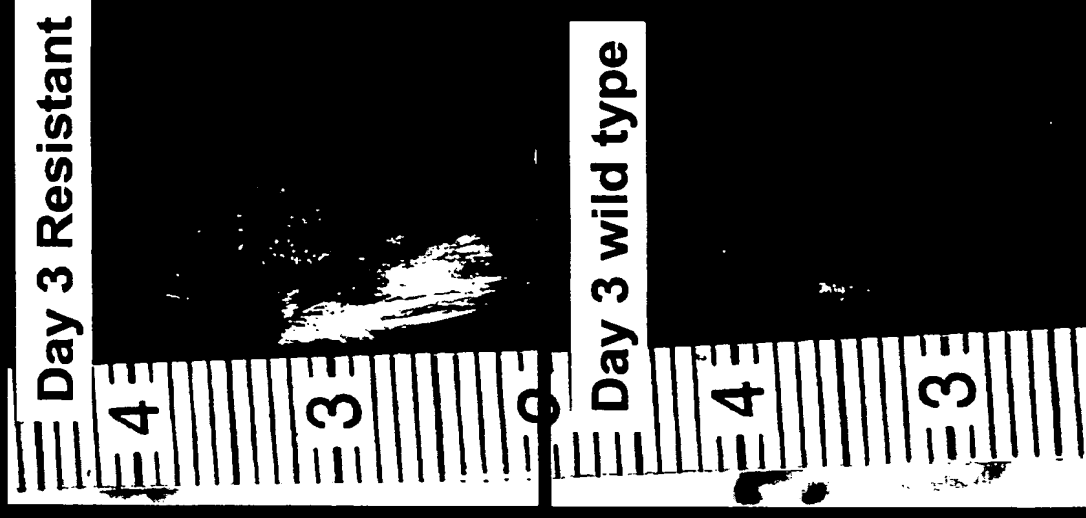
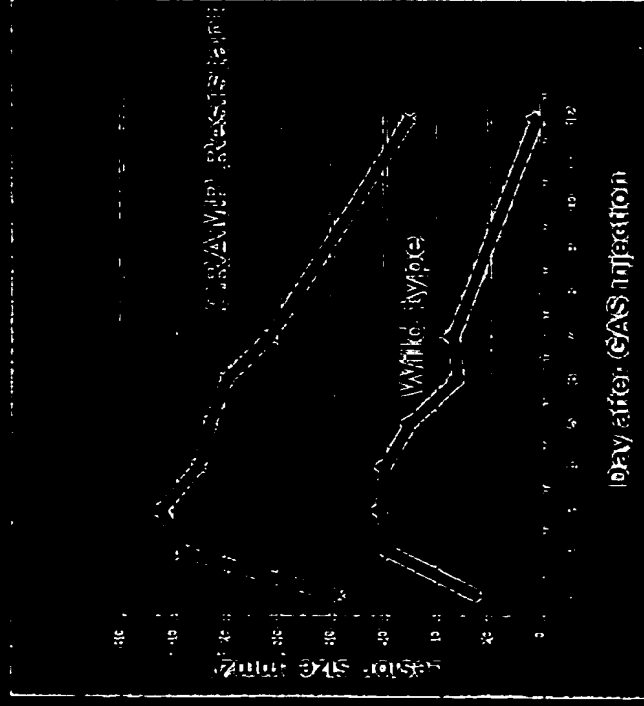
+/-

-/-



Bacteria (GAS) that are cathelicidin resistant are more pathogenic

## Lesion size

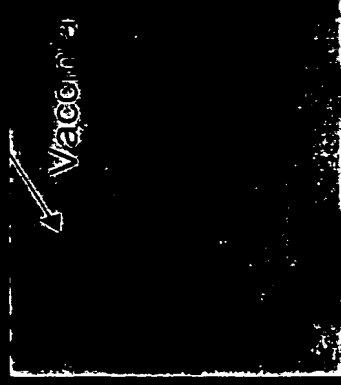


Nature, (2001) 414:454

Cathelicidins also provide defense against viral infection



No LL-37



LL-37 5  $\mu$ M

No Lesion 15/16 Control Mice

LL-37 ( $\mu$ M)	Altered virions/ virion number (%)	
0	1/23	(4%)
5	19/28	(67%)
25	27/30	(90%)

Vesicular Lesion 4/6 CRAMP --/--



### Conclusion:

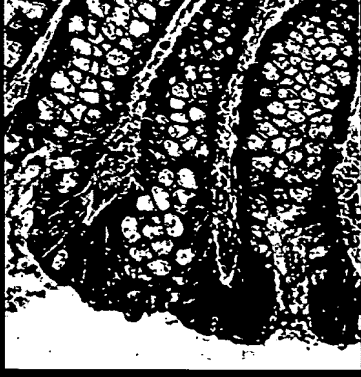
The presence of antimicrobial peptides is essential for normal defense against certain bacterial and viral skin infections

How are the cathelicidin antimicrobial peptides  
used by the skin?

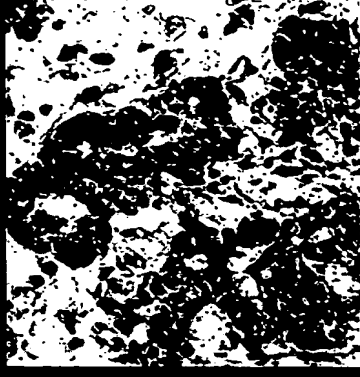
Cathelicidins are expressed at the interface



Skin



Colon



Salivary Gland



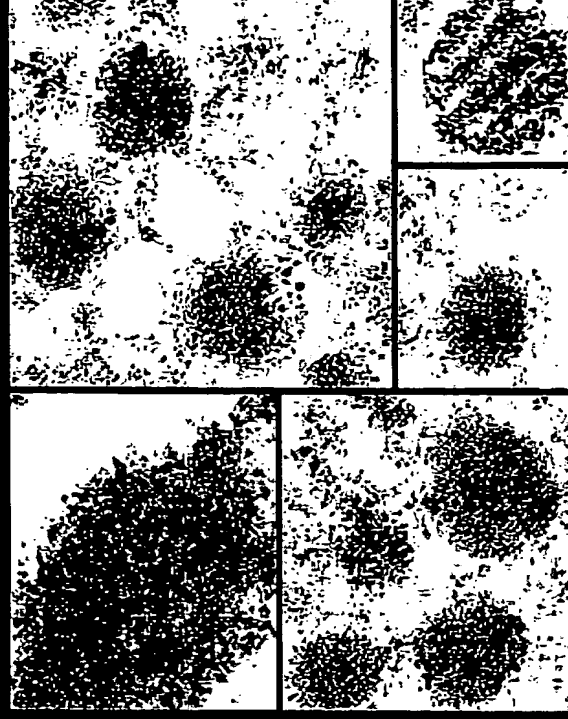
Neutrophil

Mast Cell

# Caths are stored in Keratinocyte granules

FITC Anti-LL37

Anti-LL37 immuno gold EM



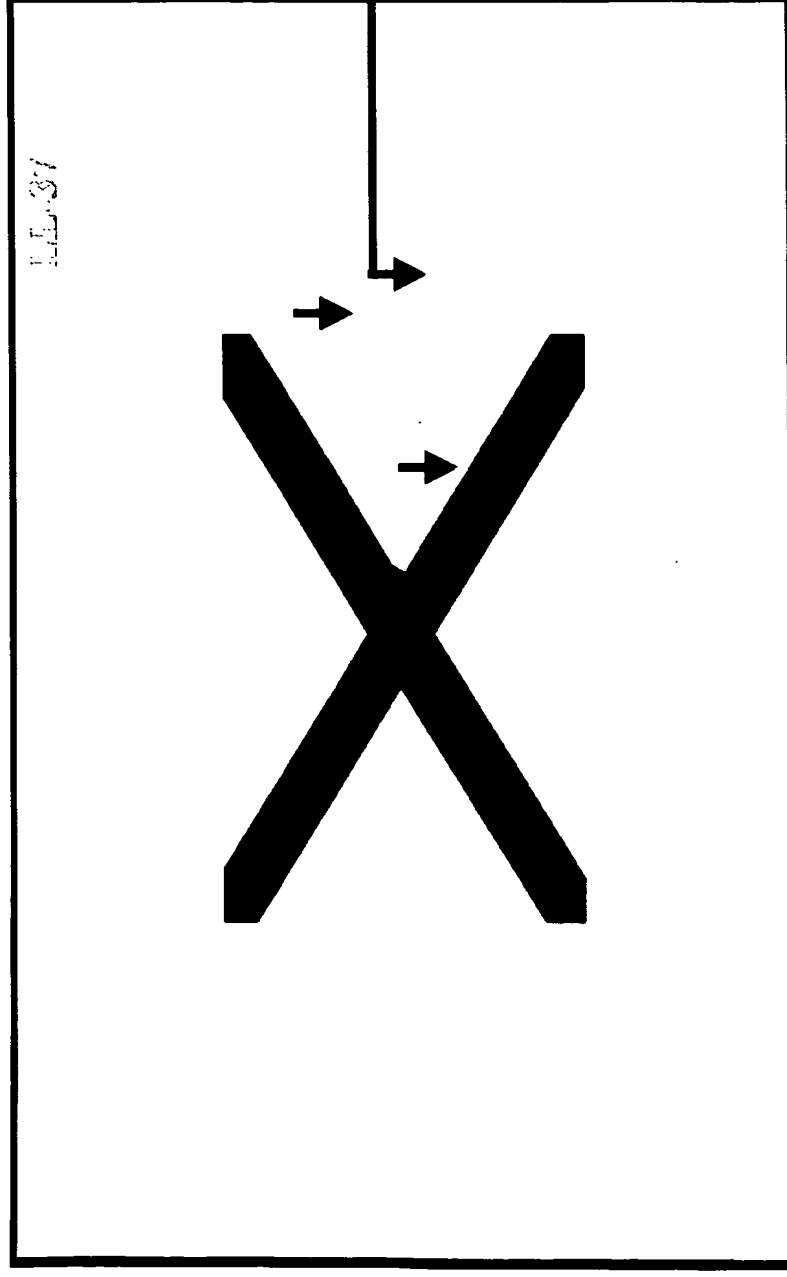
Caths are expressed in the nail apparatus



Is this an  
answer to  
normal nail  
defense?



# Cathelicidin peptides discovered in sweat with increased activity



Eccrine gland and duct  
stain for cathelicidin

New bioactive  
forms

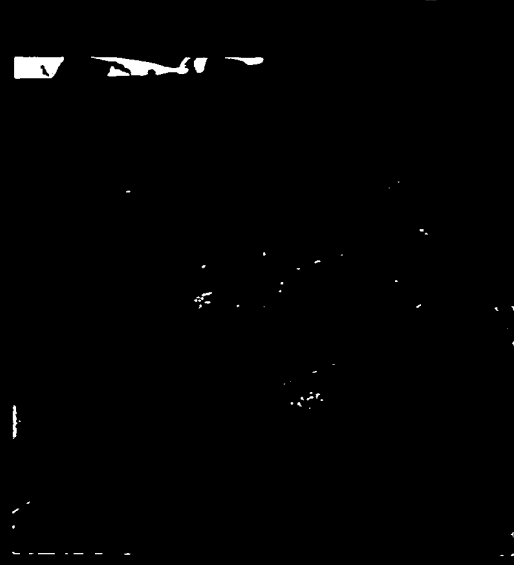
Kill bacteria  
better after  
processed by  
enzyme in sweat

Work of Dr. M. Murakami, now a Dermatology resident at Asahikawa

Does this correlate with human disease?

Atopic patients are susceptible to infection and have inflammation.  
What is their expression of antimicrobial peptides?

Impetigo



Staph  
Strep

Eczema Herpeticum



HSV, VZV

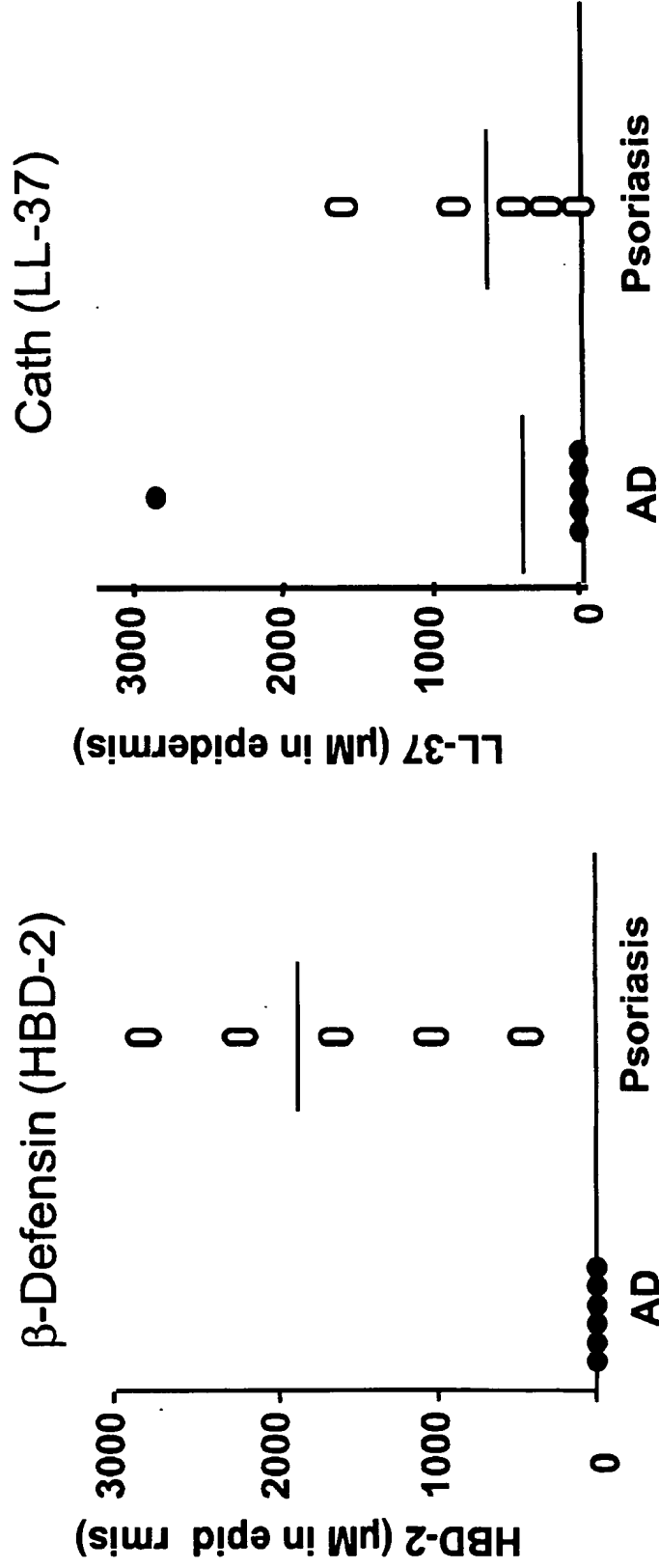
Eczema Vaccinatum



Cow pox



Compared to psoriasis, atopic patients do not respond to inflammation with a increase in antimicrobial peptides



Ong et al. (2002) N Engl J Med 347:1151-60

Work of Dr. T. Ohtake, now faculty in Medicine at Asahikawa

## Conclusions

Cathelicidins are produced by several cells in the skin.  
A lack of normal production correlates with disease

Psoriasis

Overproduction ▲

Protection

Atopic Dermatitis

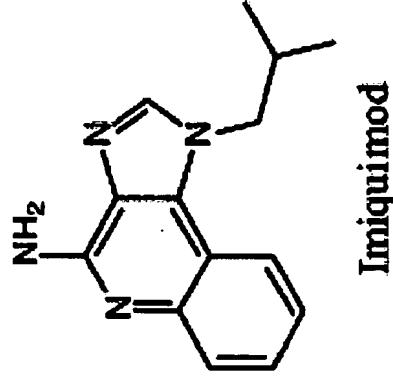
Underproduction ▼

Infection

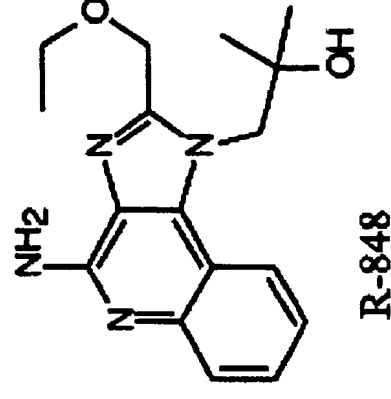


Can our increased understanding of innate immunity  
and antimicrobial peptides be used for therapy?

## Imiquimod:



Imiquimod



R-848

Stimulus of TLR-7 provides useful pro-inflammatory tool.

Effective for:

Common warts, condylomata, molluscum,  
BCC, Bowens, Bowenid papulosis, AK, lentigo maligna,  
stucco keratosis, Porokeratosis of Mibelli

## Antimicrobial peptides have been successfully used in preliminary animal models

Protection of burn wounds against infection (2001) Crit Care Med 29:1431	Skin wounds (2001) Blood 97:297
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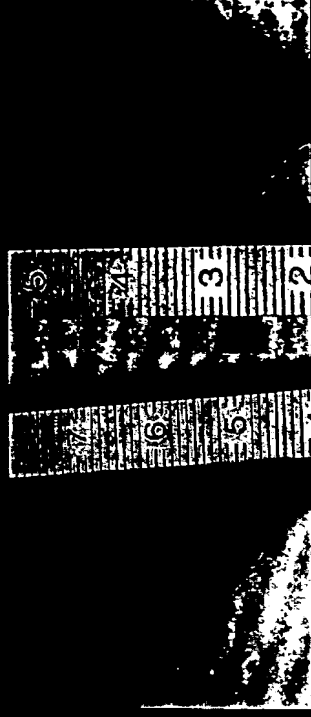
# Antimicrobial gene therapy is effective in mice

PR-39 expression in wound lesion



anti-PR-39

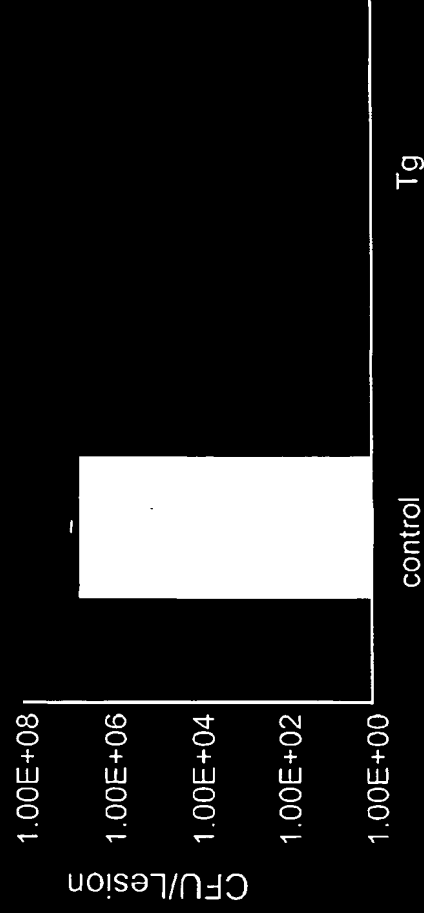
pre-immune



control

Tg

Live bacteria in lesion (day6)



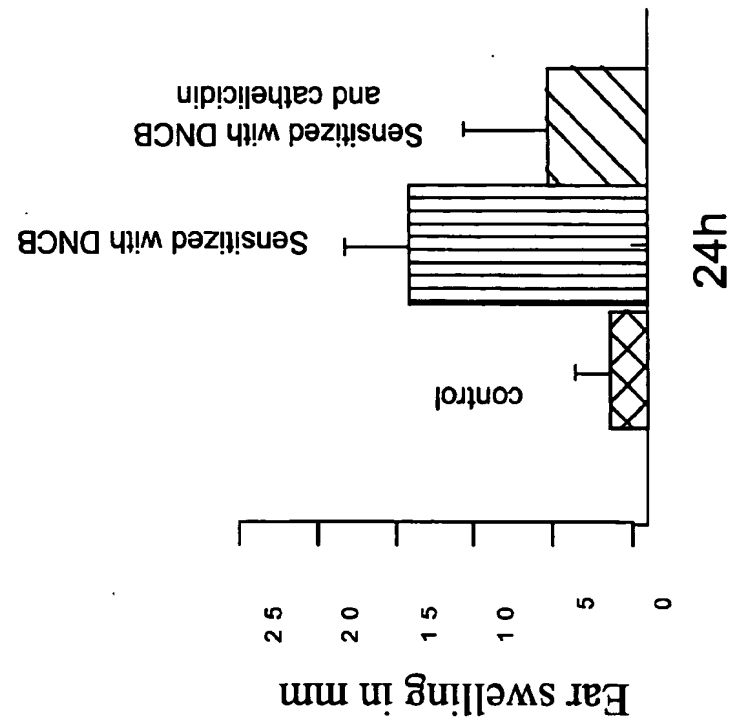
## Human trials are underway

<u>Route</u>	<u>Peptide</u>	<u>Company</u>	<u>Application</u>	<u>Stage</u>
Topical	MSI-78	Maganin	DM ulcers	Phase 3
Topical	MBI-226	Microligix	Cath infections	Phase 3
Topical	MBI-594		Ame	Phase 2
Oral	Protegrin	Intrabiotics	Mucositis	Phase 3
Oral	Histatin	Demegen	Gingivitis	Phase 2
Sysemic	Helicomyacin	Entomed	Antifungal	Pre-clinical
Sysemic	Lactoferrin	AM Pharma	Antibacterial	Pre-clinical
Sysemic	BPI	Xoma	Meningitis	Phase 3

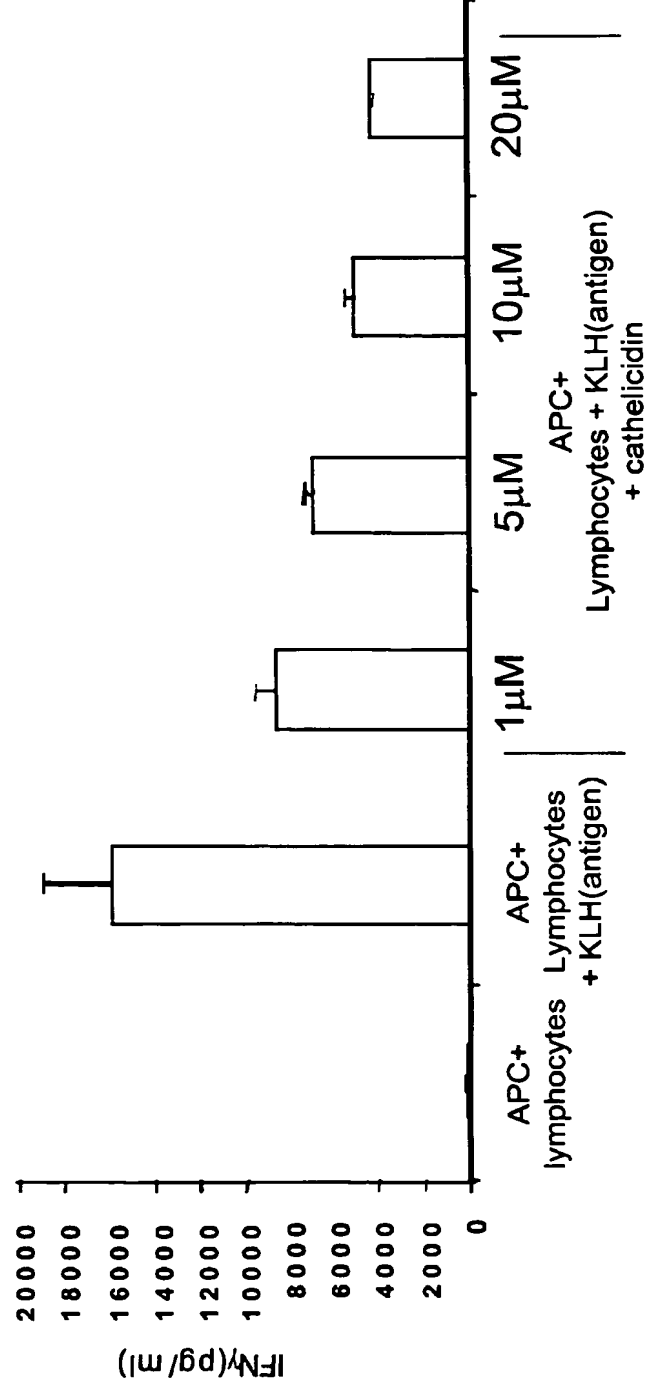
However,  
Antimicrobial Peptides do more than kill microbes!



Cathelicidin blocks allergic contact dermatitis  
in mice



## Cathelicidin blocks antigen presentation



But chronic administration of cathelicidin is proinflammatory

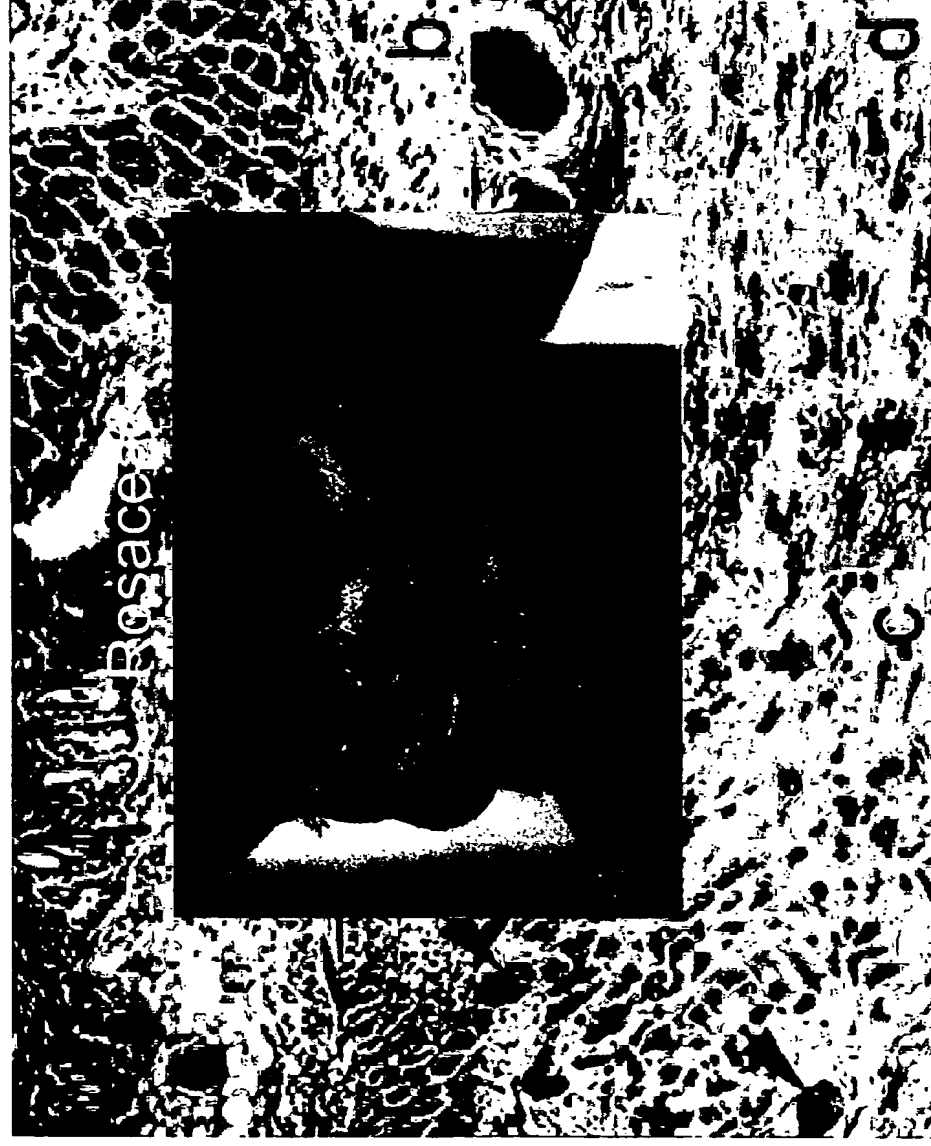
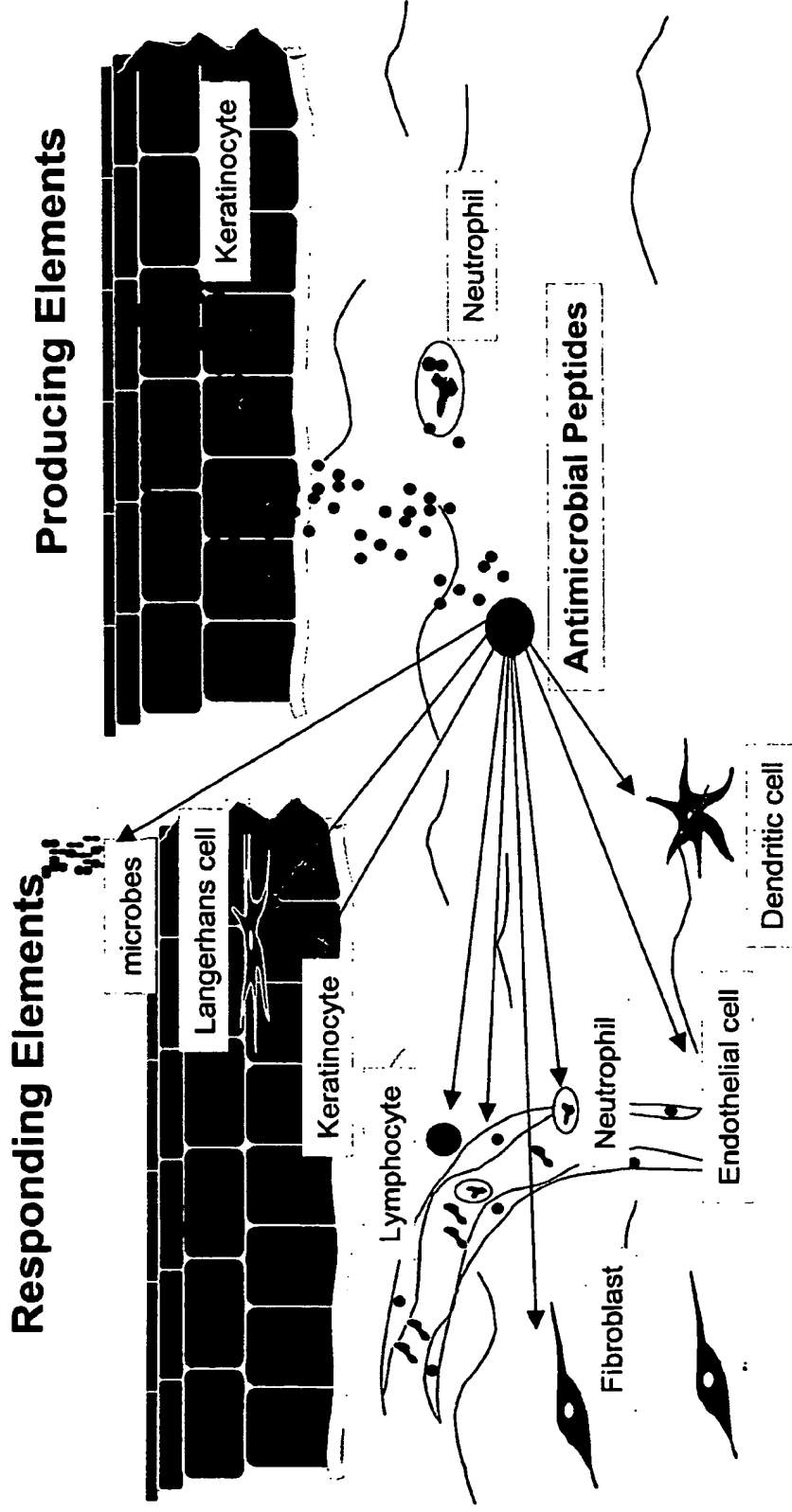


Figure 5

- Antimicrobial peptides are important to innate immune defense
- Defects in innate immunity may explain certain skin diseases
- Action of antimicrobial peptides are part of the whole immune system

# SUMMARY:

## Antimicrobial Peptides in the Skin



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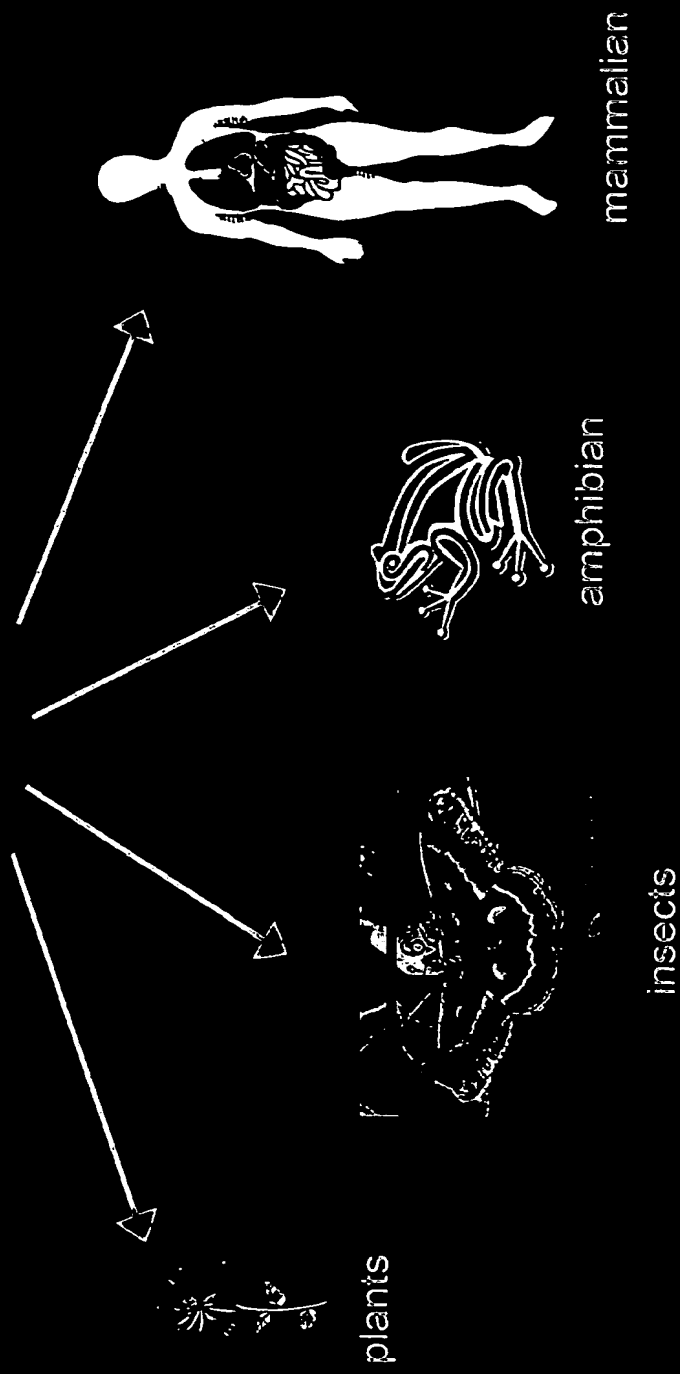
FIGURE 19 - 37pg

# Antimicrobial Peptides and Innate Defense of the Skin

Richard L. Gallo, M.D., Ph.D.  
Medicine and Pediatrics  
U. of California, San Diego



Peptides with antibiotic activity are found throughout nature.





Over **800** peptides now known  
Searches based on ability to kill microbes in culture

Multiple antimicrobial peptide gene families (partial list)

Bombinins	Defensins
amphibians	plants
Cecropins	insects
insects	mammals
mammals	
Dermaseptins	Cathelicidins
amphibians	mammals
Magainins	fish
amphibians	

[www.bbcm.univ.trieste.it/~tossi/pag1.htm](http://www.bbcm.univ.trieste.it/~tossi/pag1.htm)

Antimicrobial peptides are often cationic and amphipathic



Human alpha defensin



Frog Maganin

+ Red = basic amino acids  
+ Green=hydrophobic

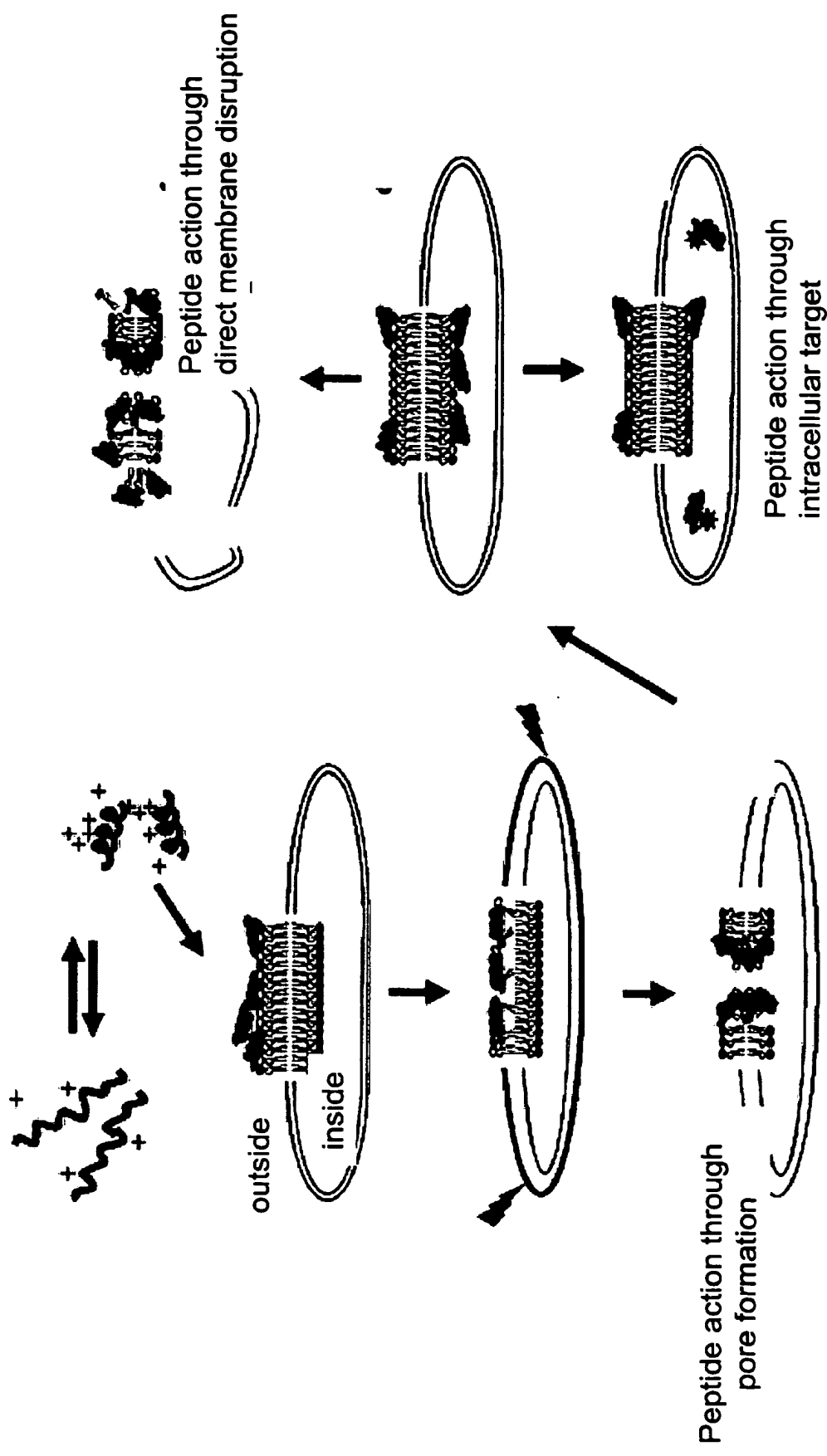


Pig Cathelicidin  
(Indolicidin)



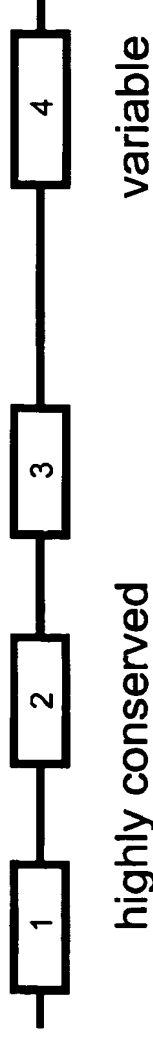
Pig Cathelicidin  
(Protegrin)

# Antimicrobial action is dependent on membrane activity



# Cathelictidins:

Cathelictidin  
Gene organization



elastase or  
proteinase-3



~30 aa

signal  
peptide

94-144 aa

cathelin prosequence domain

12 -100 aa

mature amp

Full-Length  
Cathelictidin  
Precursor



enriched in  
specific a.a.  
like pro/arg  
(e.g. PR-39)



beta-sheet  
structure  
(e.g. protegrin)



amphipathic  
alpha-helix  
(e.g. II--37)

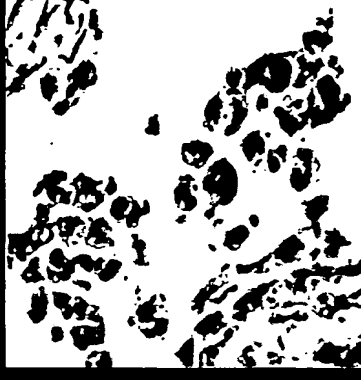
# Caths are expressed at the interface



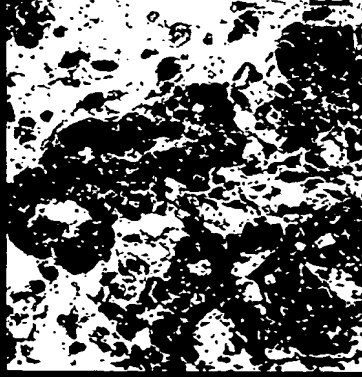
Skin



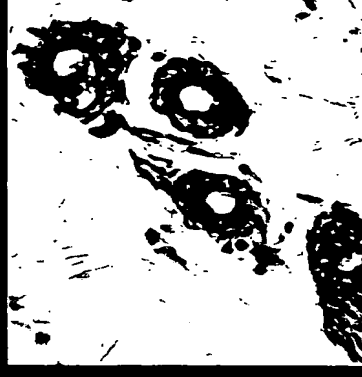
Colon



Neutrophil



Salivary Gland



Sweat Gland

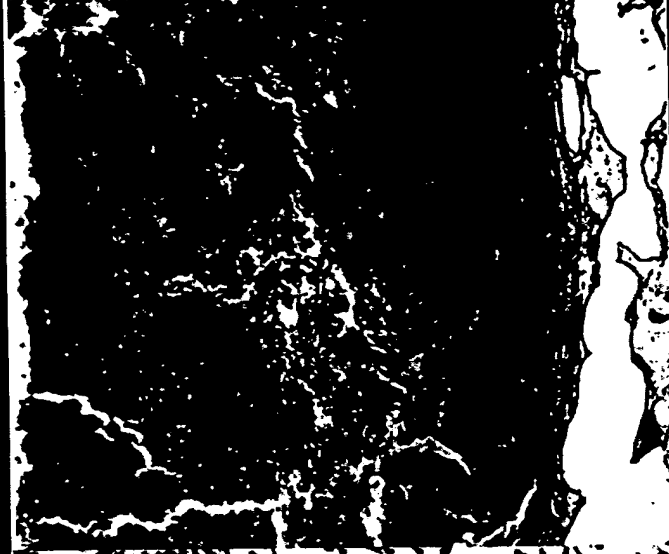
Mast Cell

## In Skin, Injury or Infection Triggers Cath Expression

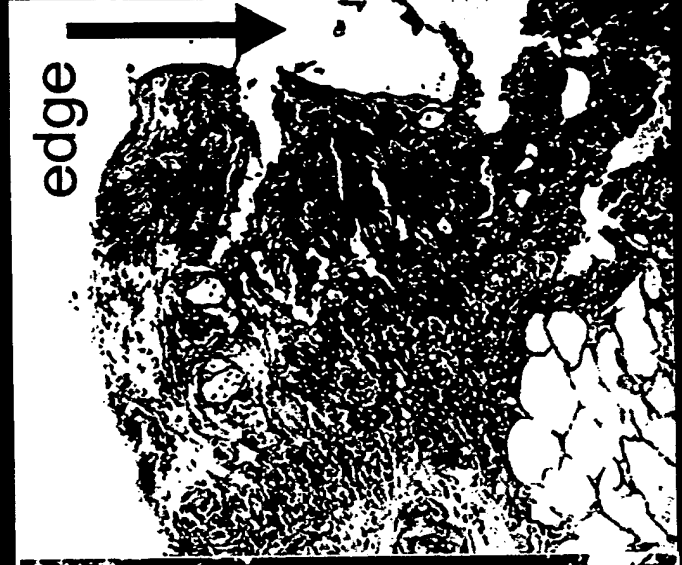
Normal skin



Strep infection

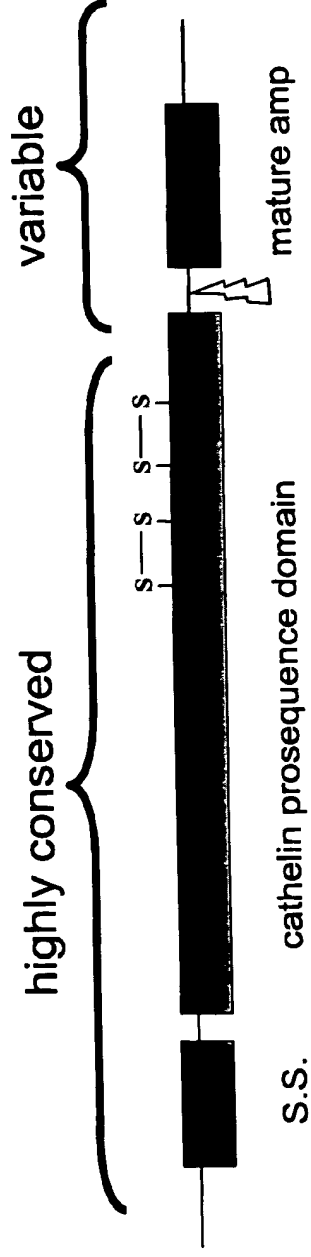


12 hr Wound



Brown staining= cathelicidin peptide immunoreactivity

# Cathelictidins: Prodomain Function?



After Ni  
+IPTG  
column



Human  
cathelin

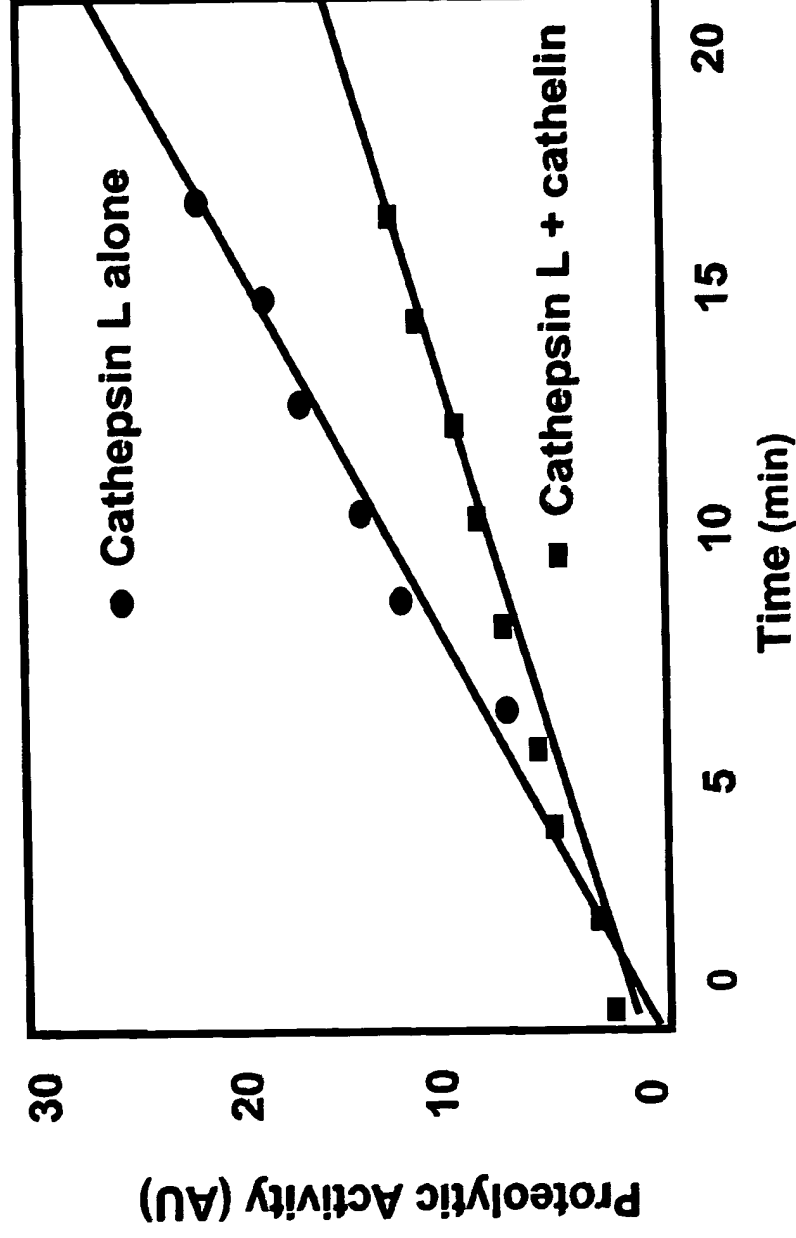
Human cathelin Domain



Recombinant expression in *E. coli*

# Cathelidins: Prodomain Function

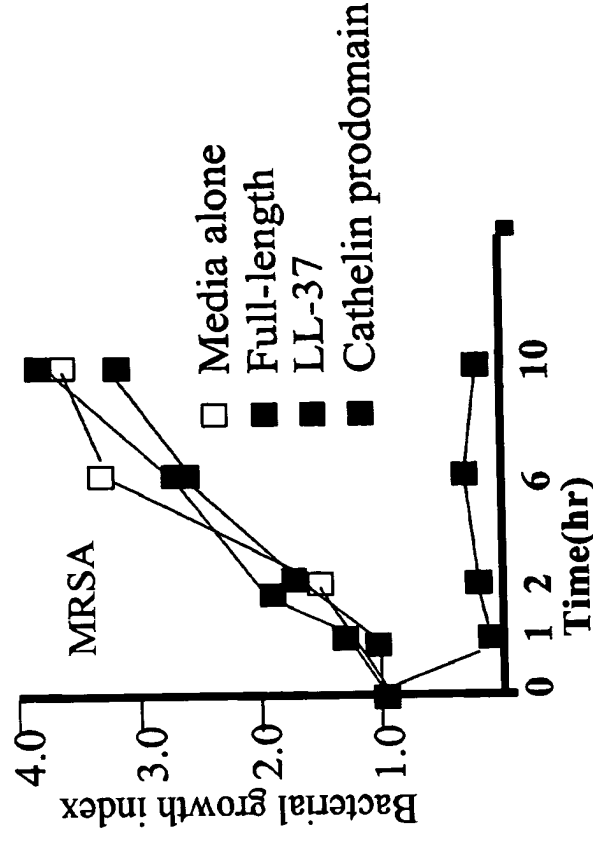
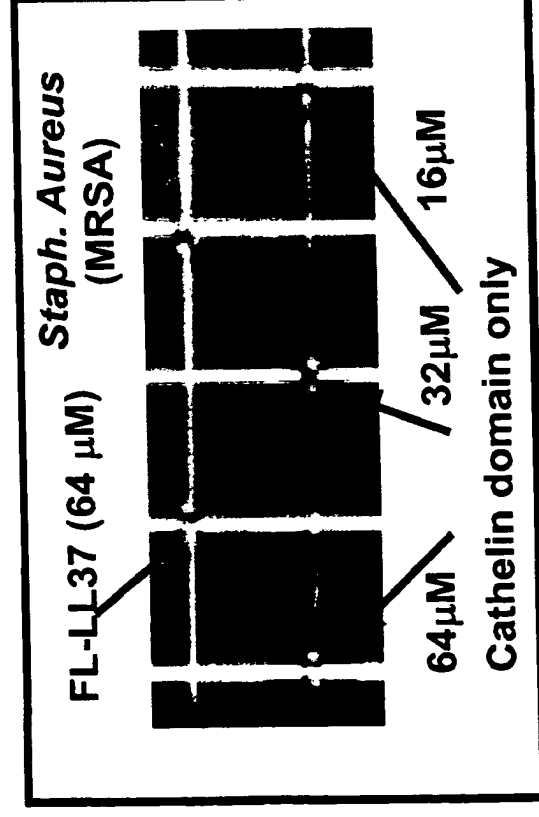
---



Human cathelin domain has Cysteine Protease Inhibitor activity



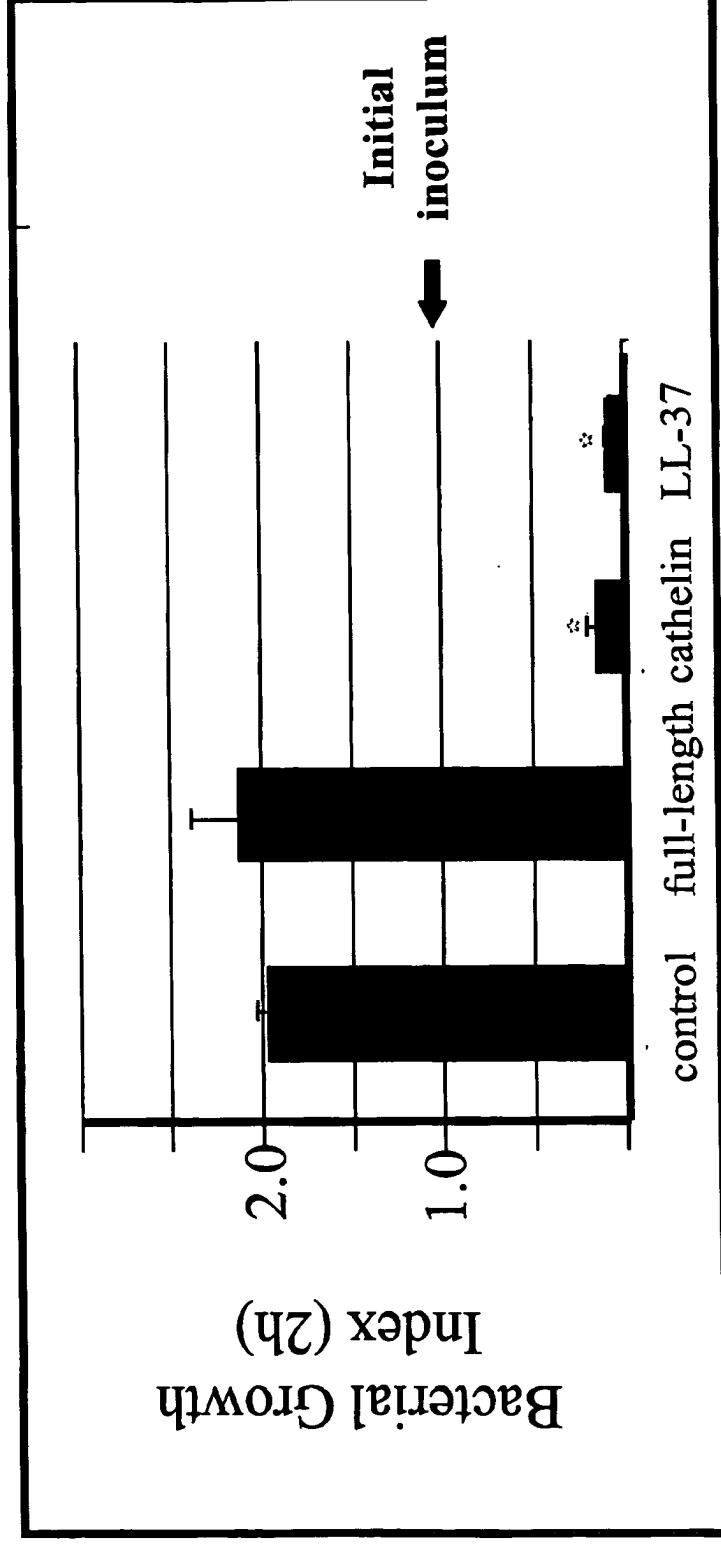
# Cathelcidins: Prodomain Function



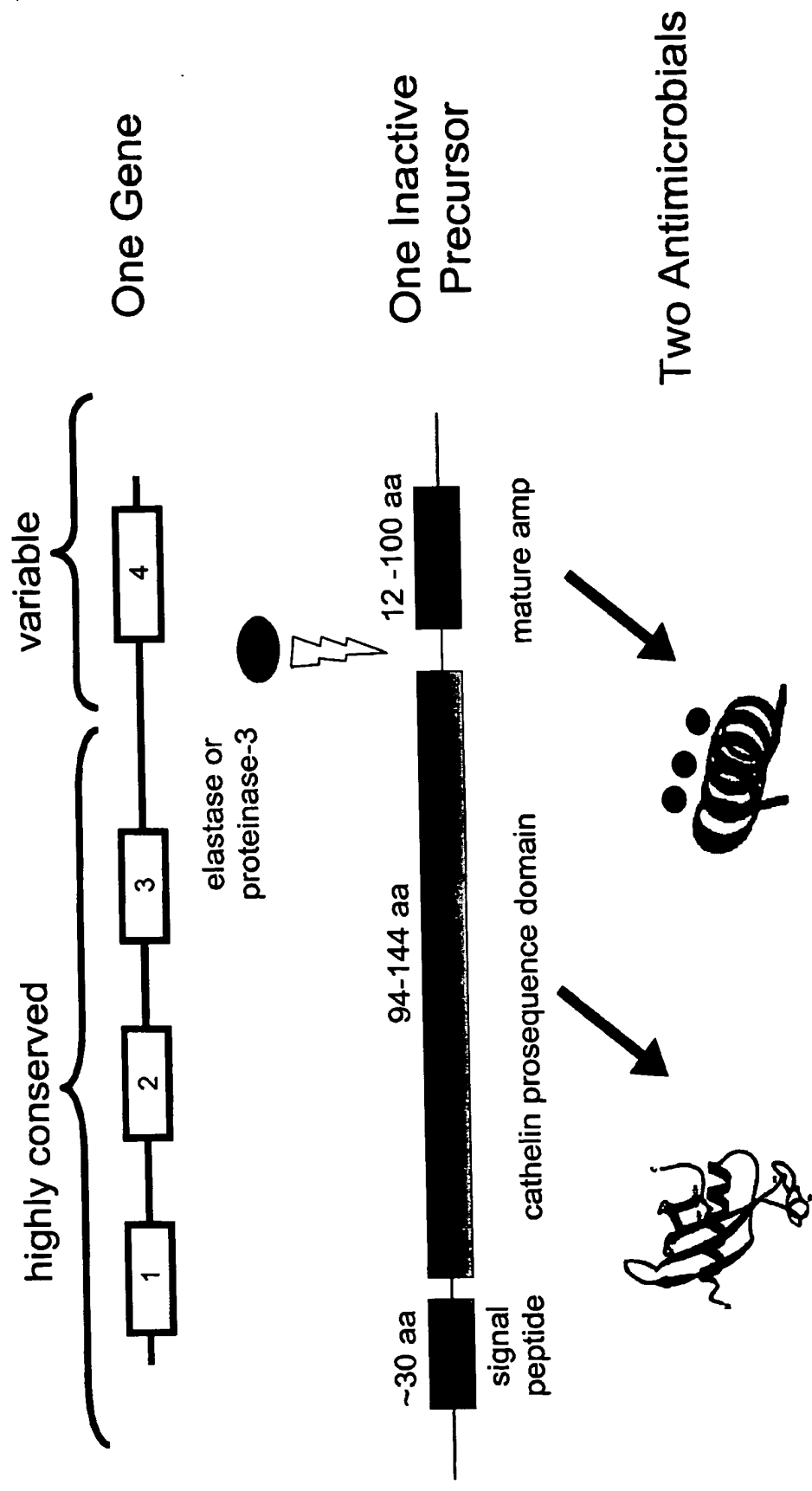
Human cathelin domain has independent antimicrobial activity

## Keratinocytes with expressed cathelicidin gain function

HaCat human keratinocyte cell line  
Lentiviral expression system  
Full-length, cathelin prodomain and LL-37 C-terminal AMP  
Test bacterium: Cathelicidin-sensitive *S. aureus* mprF mutant

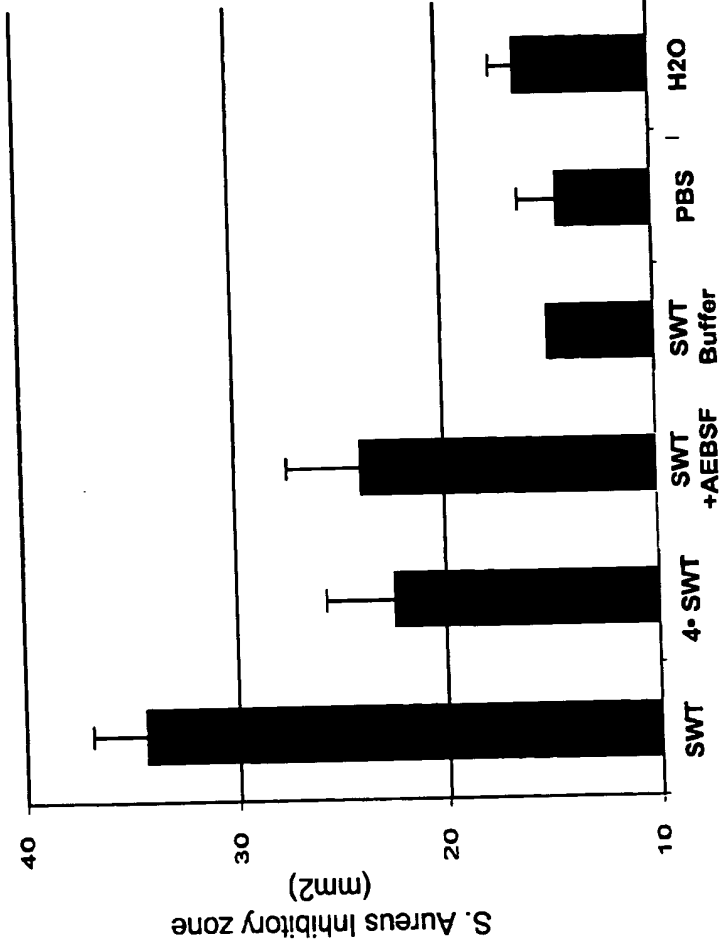
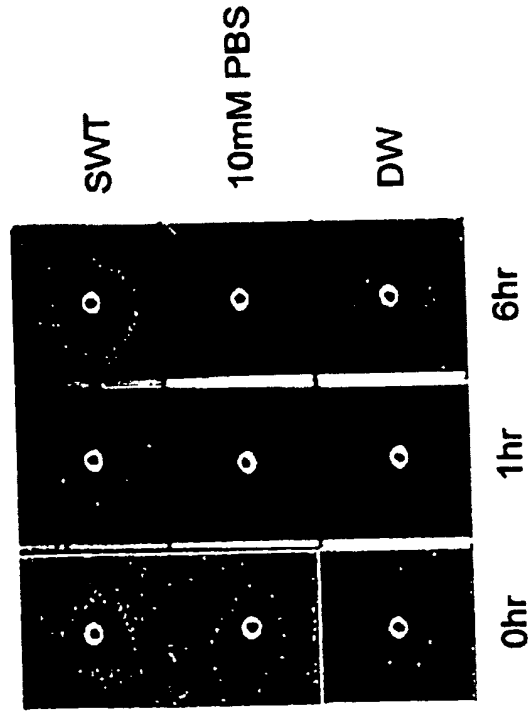


# Cathelicidin: "Double Trouble" for Bacteria

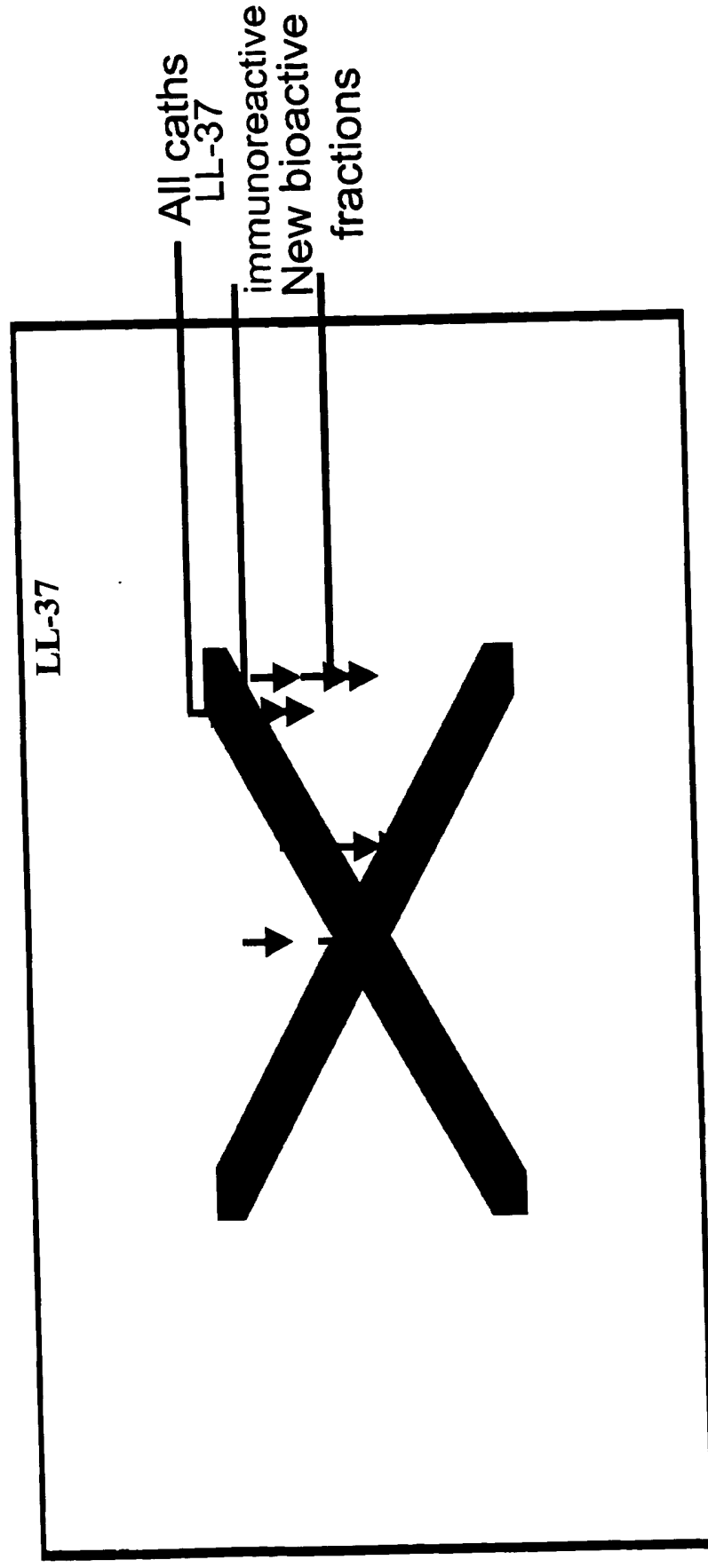


# Human cathelicidin LL-37 increases activity at the skin surface

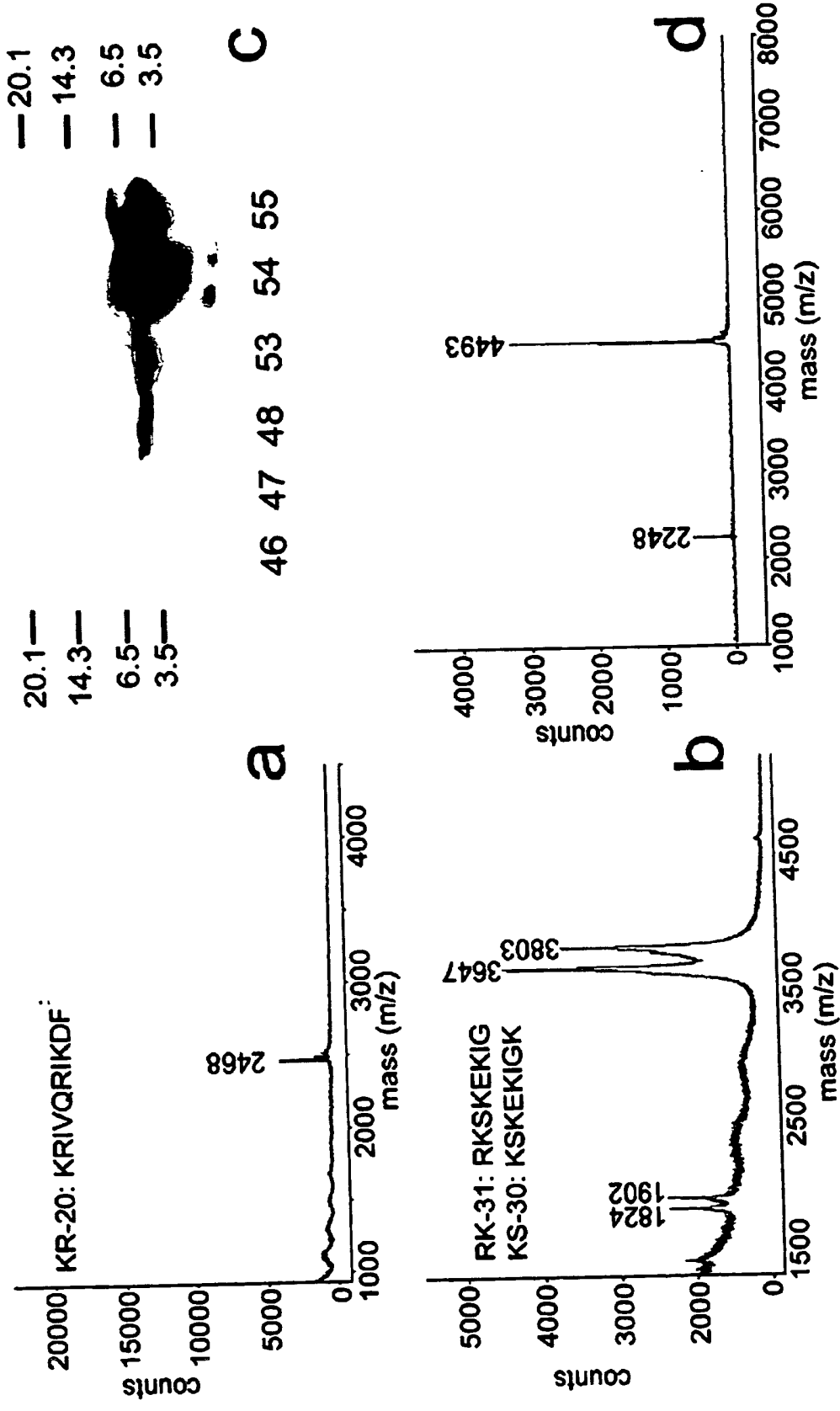
*Staph aureus* Mprf



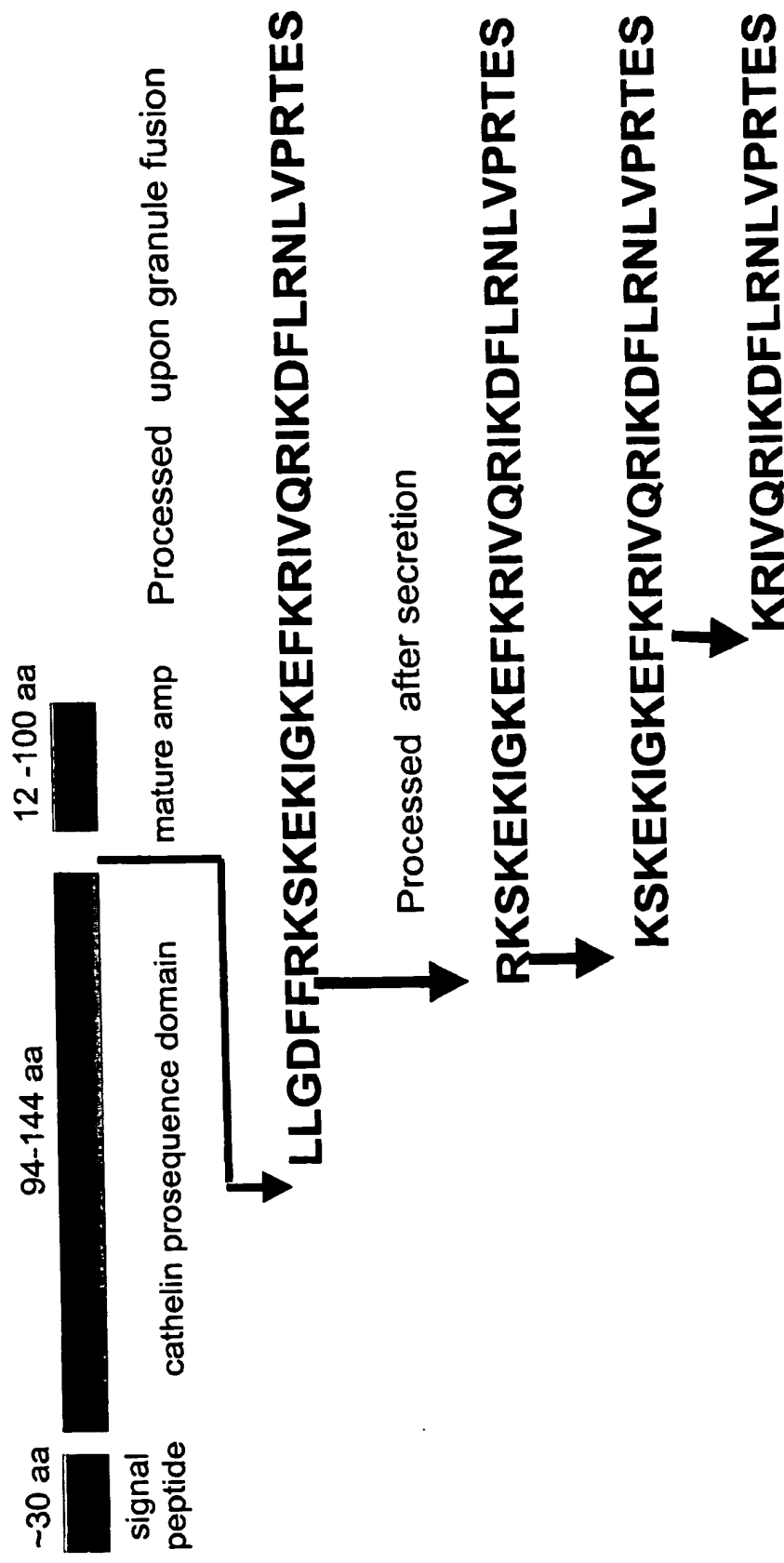
# Detection of Cathelicidin peptides on the skin surface



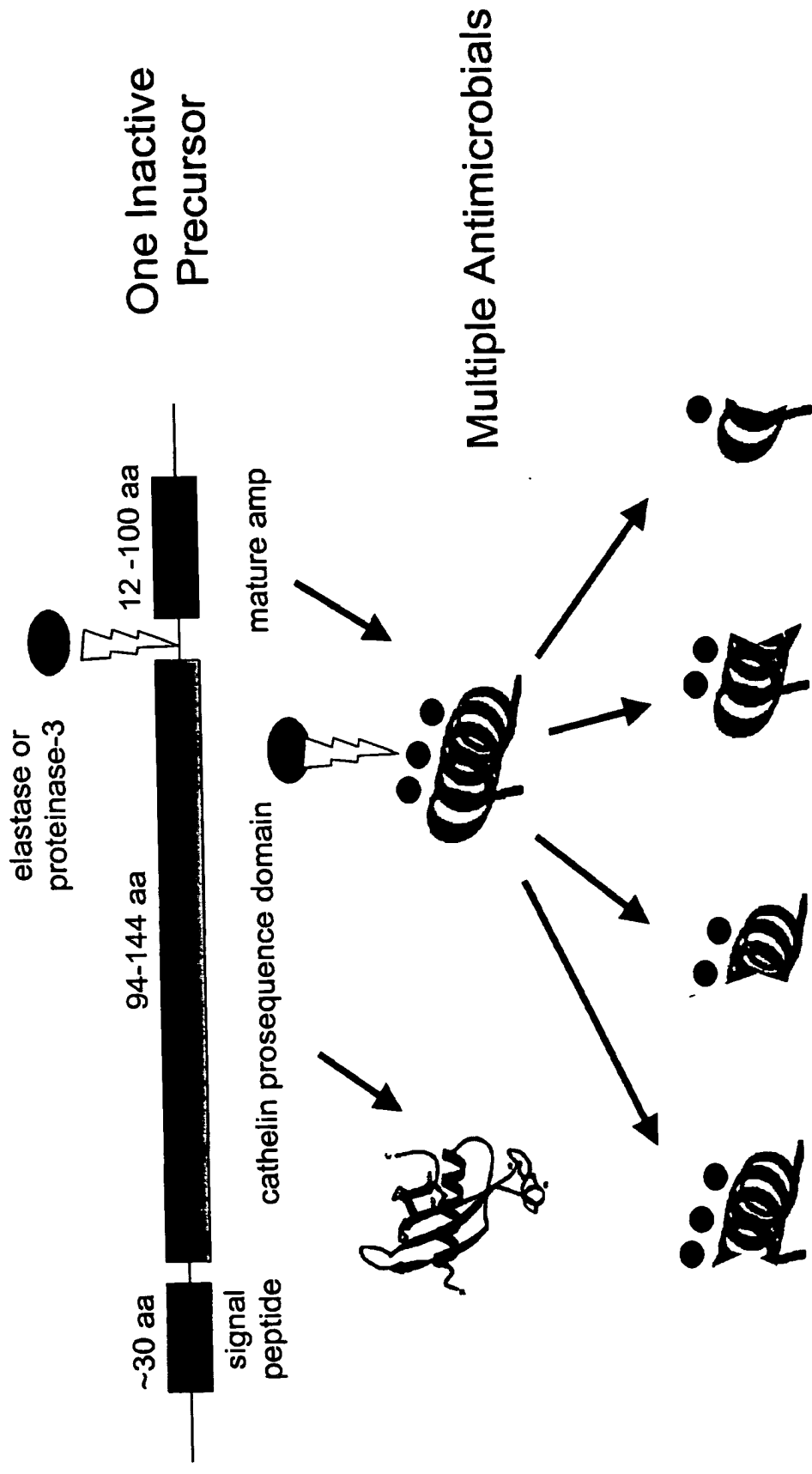
# Identification of cathelicidin peptides on the normal skin surface



## Processing of cathelicidin peptides on the normal skin surface



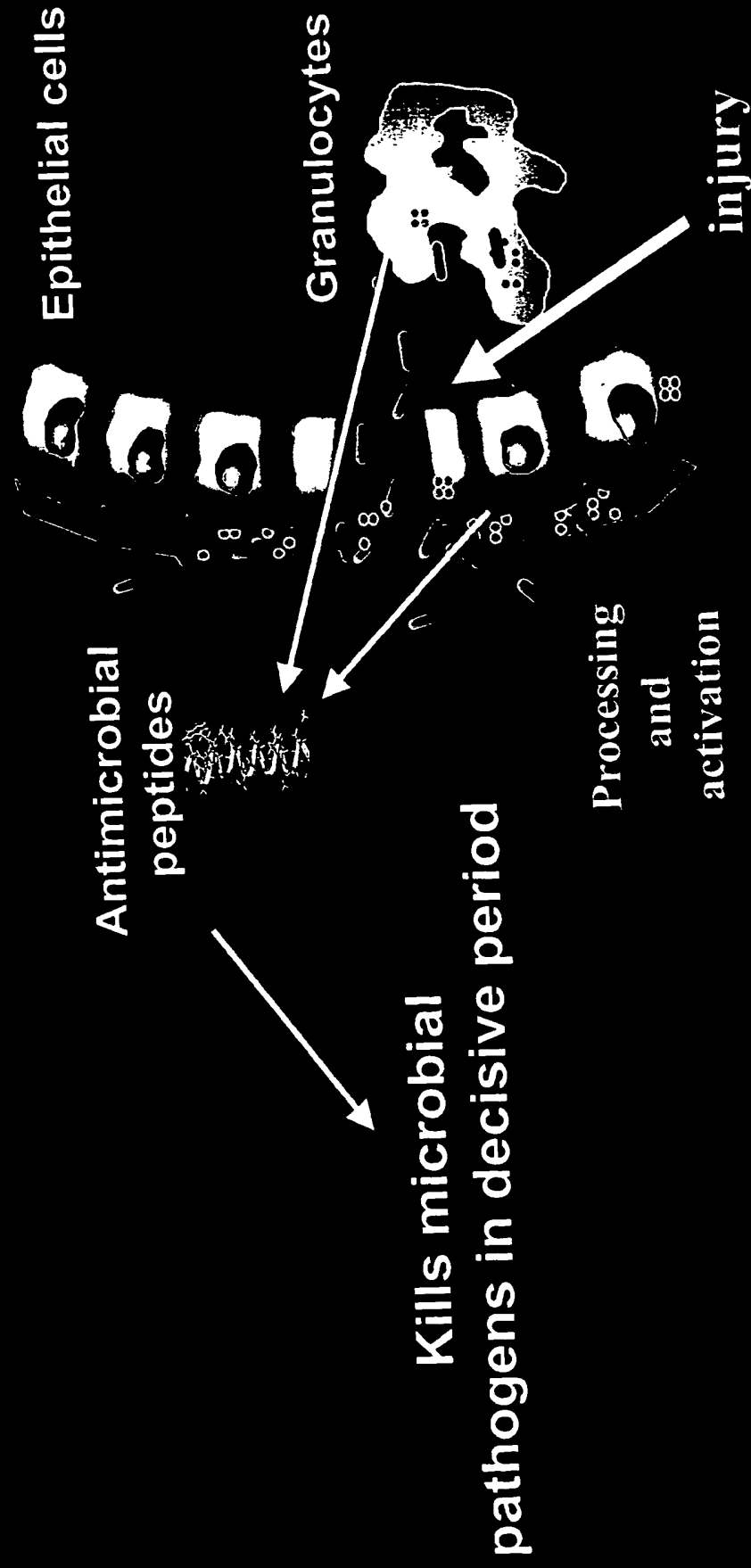
# Cathelicidin: Single Gene , Many Antibiotics





### A Simple Hypothesis:

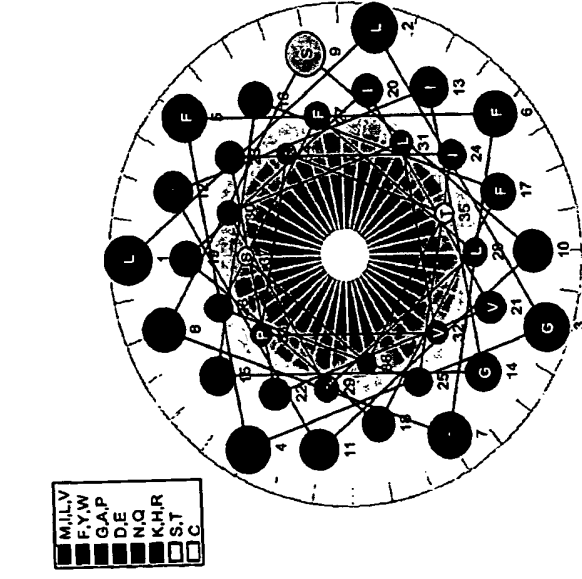
Expression patterns, response to injury, antimicrobial action all support a model of innate immune defense



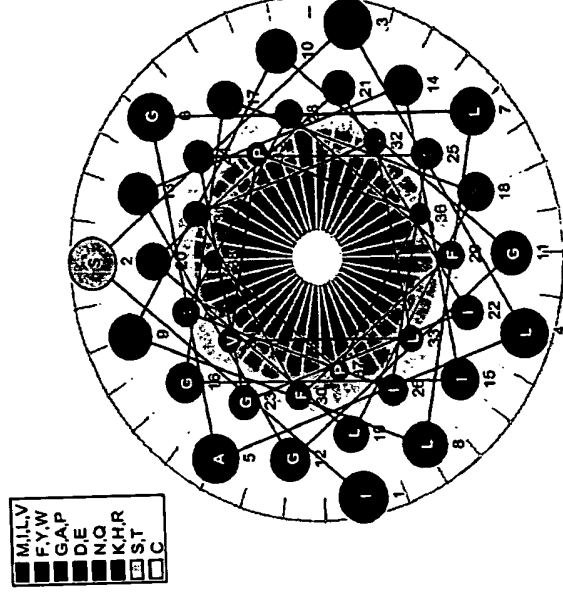
Is this true?

# Cathelicidin Peptides: Man vs. Mouse

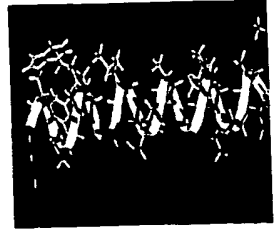
LL-37 (Human)



CRAMP (Mouse)



Similar Encoding Genes  
Similar Alpha-Helical Structure  
Similar Tissue Distribution  
Similar Spectrum of Activity

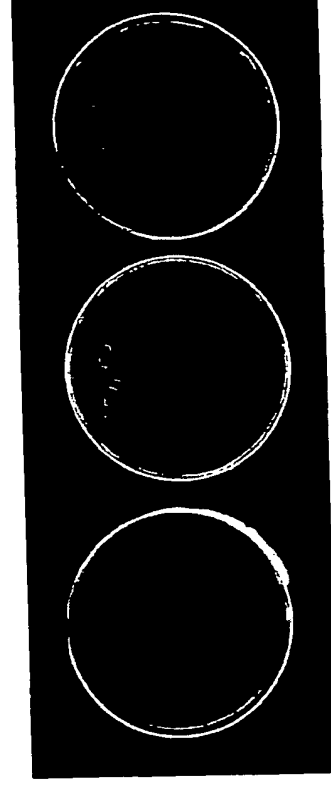


Cathelicidin deficient mice are more susceptible to  
Invasive Group A *Streptococcus*

**+/- day 7**



**-/- day 7**



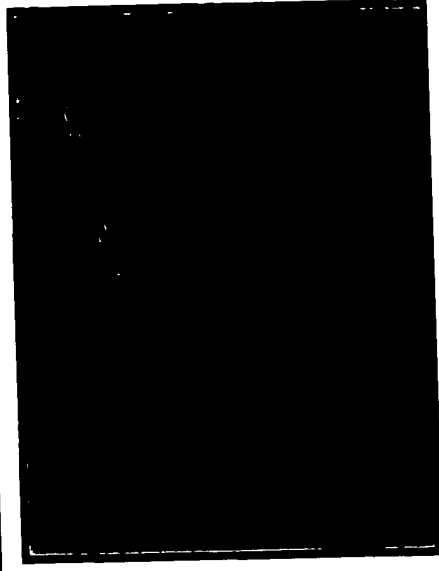
# Activity against vaccinia supports role for Caths in immune defense against viral infection



No LL-37

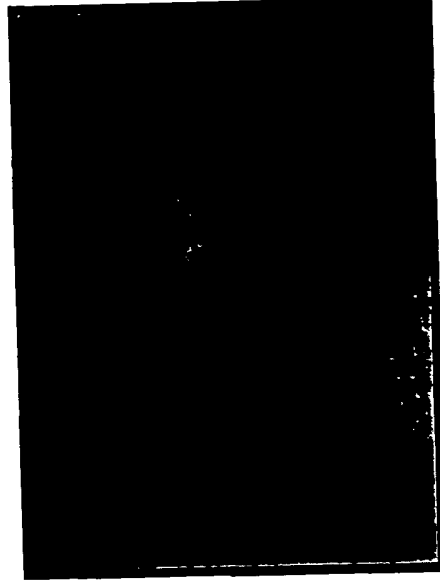


LL-37 5 uM



No Lesion 15/16 Control Mice

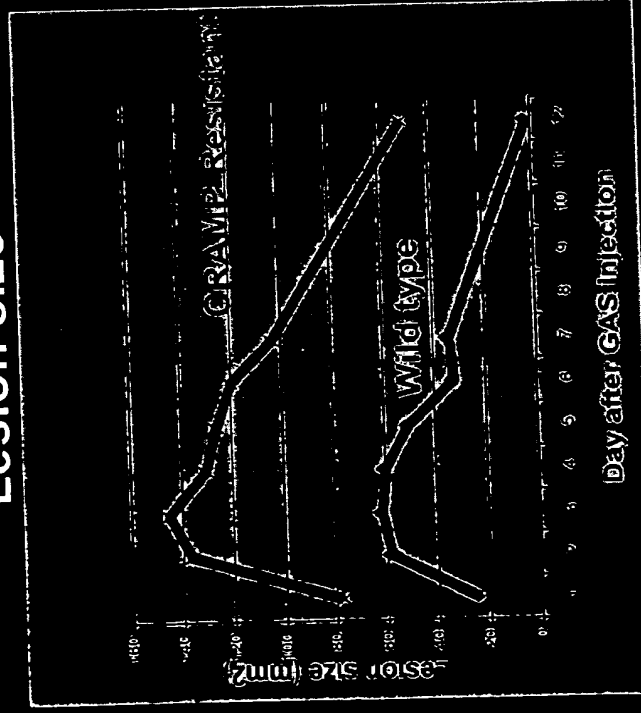
LL-37 ( $\mu$ M)	Altered virions/ virion number (%)
0	1/23 (4%)
5	19/28 (67%)
25	27/30 (90%)



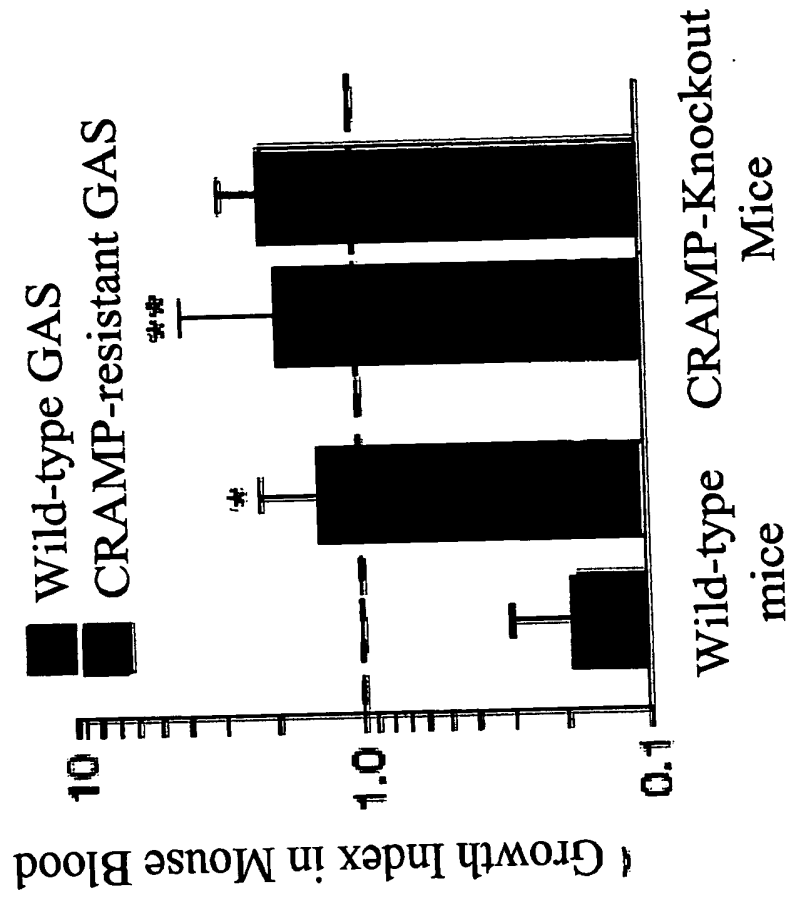
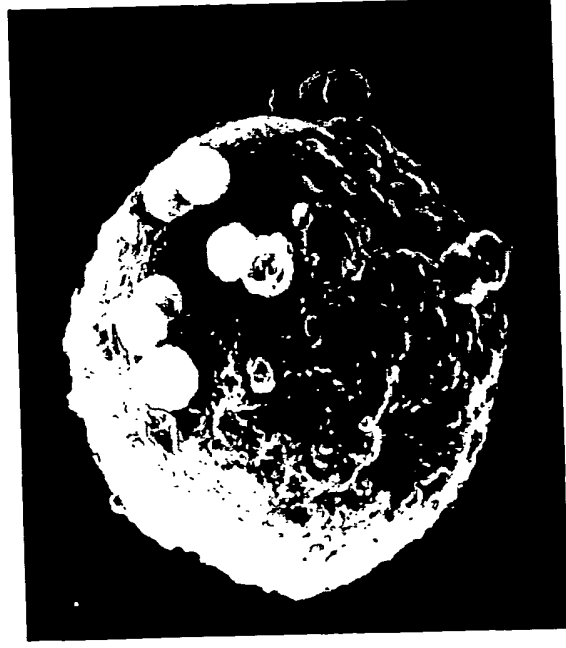
Vesicular Lesion 4/6 CRAMP --/--

Bacteria (GAS) that are cathelicidin resistant are more pathogenic

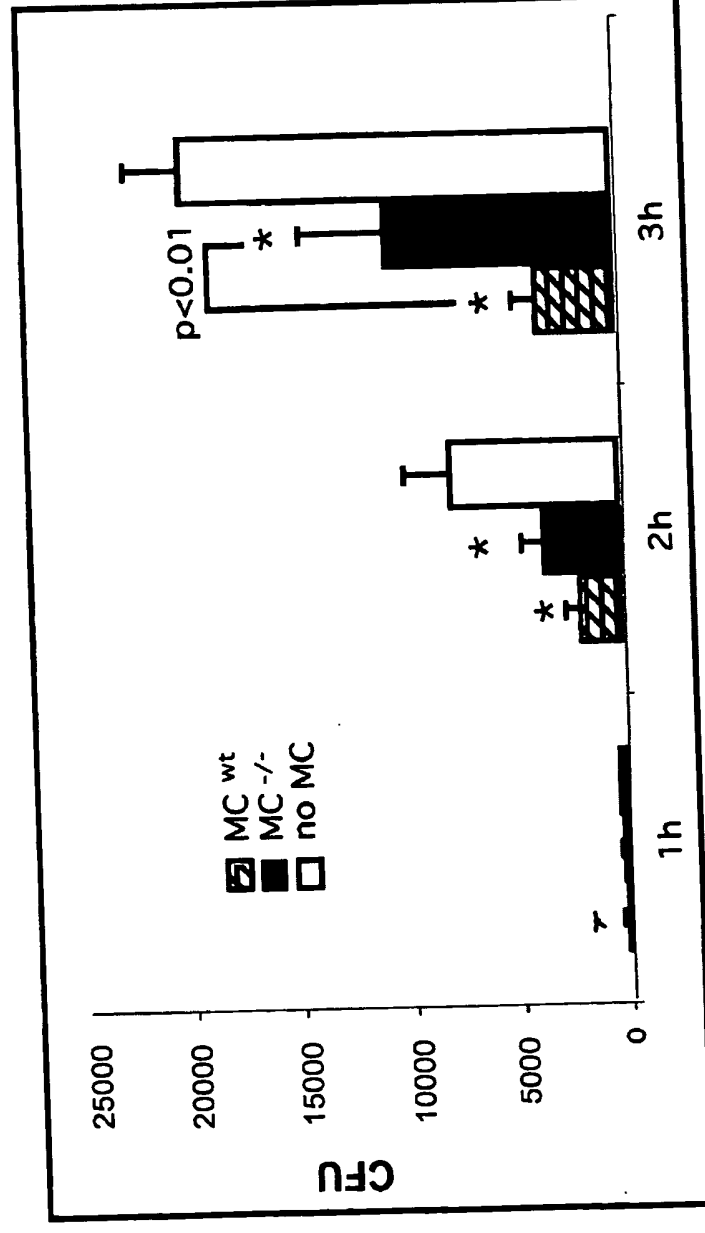
### Lesion size



# Cramp Effect Evident in CELL-SPECIFIC assay Blood-Killing Assay



# Cramp Effect Evident in CELL-SPECIFIC assay Mast Cells

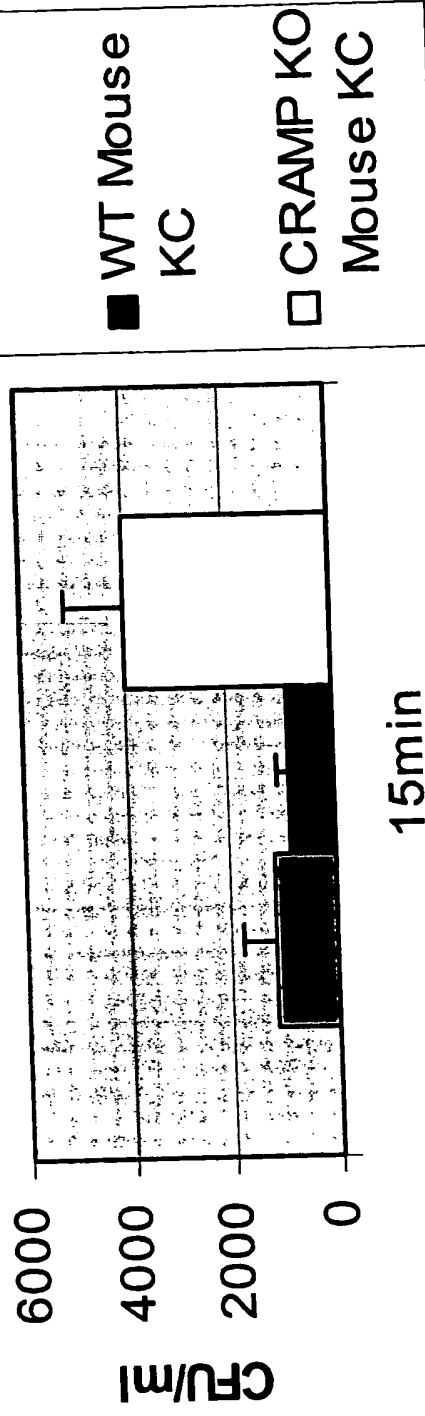




# Cathelicidin Effect Evident in CELL-SPECIFIC assay

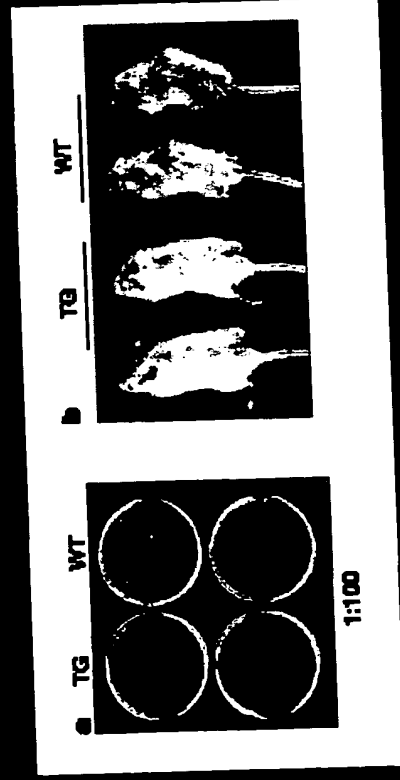
## Keratinocytes

Killing of *S. aureus* mprF by  
keratinocytes



# Immune protection by heterologous antimicrobial peptide over-expression.

Human antimicrobial peptide expressed in mice paneth cells confers resistance to *Salmonella typhimurium*



Amphibian antimicrobial peptide expressed in tabacum confers resistance to *Speudomonas syringae* or *P. nicotianae*



*Nature*, 2003 422 Apr 3

*Biochem.J* 2003, 370

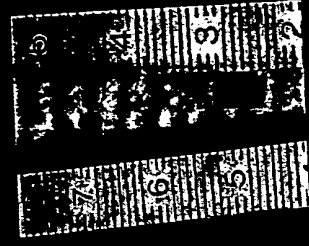
# Immune protection by heterologous antimicrobial peptide over-expression.

PR-39 expression in skin



anti-PR-39

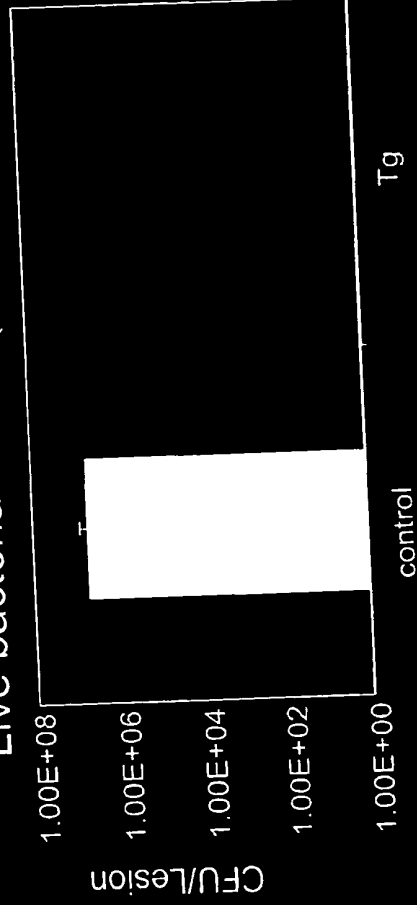
pre-immune



control

Tg

Live bacteria in skin (day6)

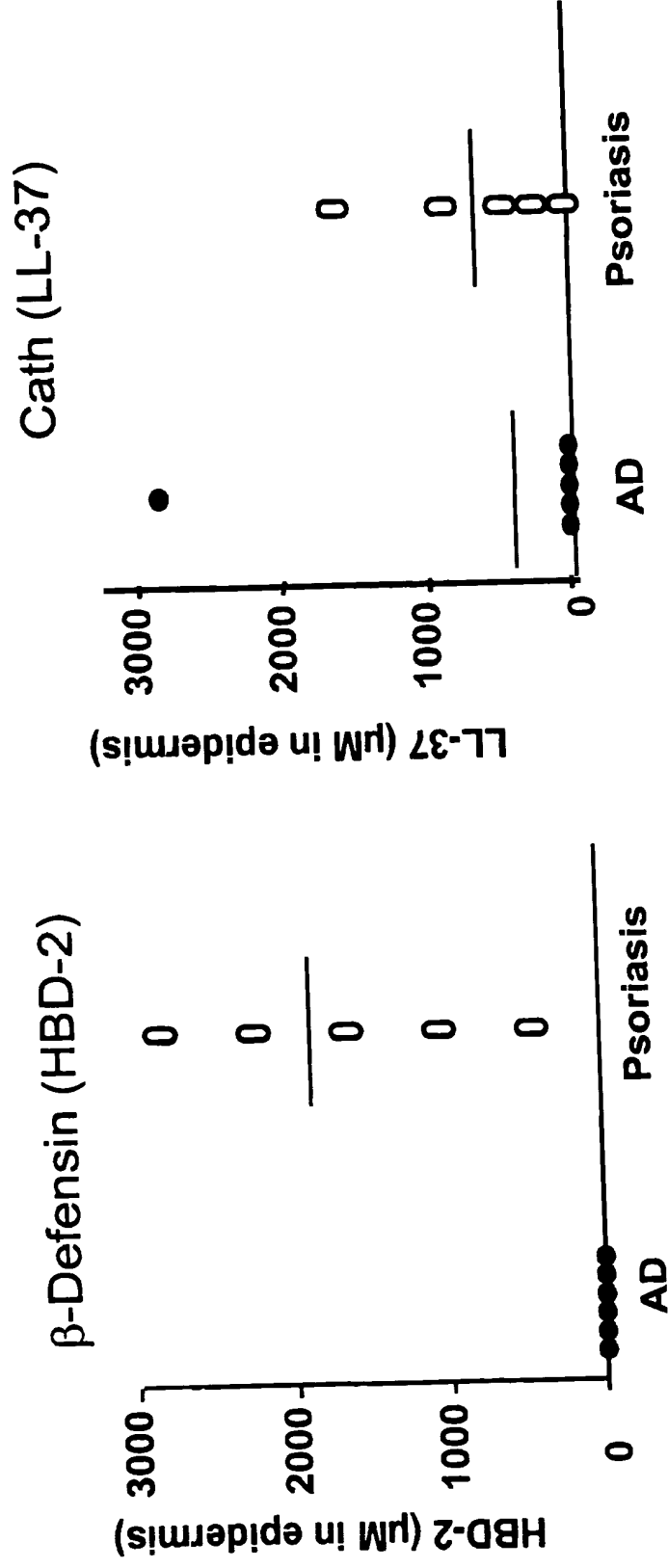


T. Ohtake

So why do the antimicrobial  
Peptides fail us?



## Th2 cytokines in Atopic Dermatitis inhibit antimicrobial peptide induction



# Consequences of a lack of antimicrobial peptide expression?

Eczema Herpeticum



HSV, VZV

Eczema Vaccinatum



Cow pox

Impetigo

Staph  
Strep

**Recall**  
These peptides have diverse activity on the host.

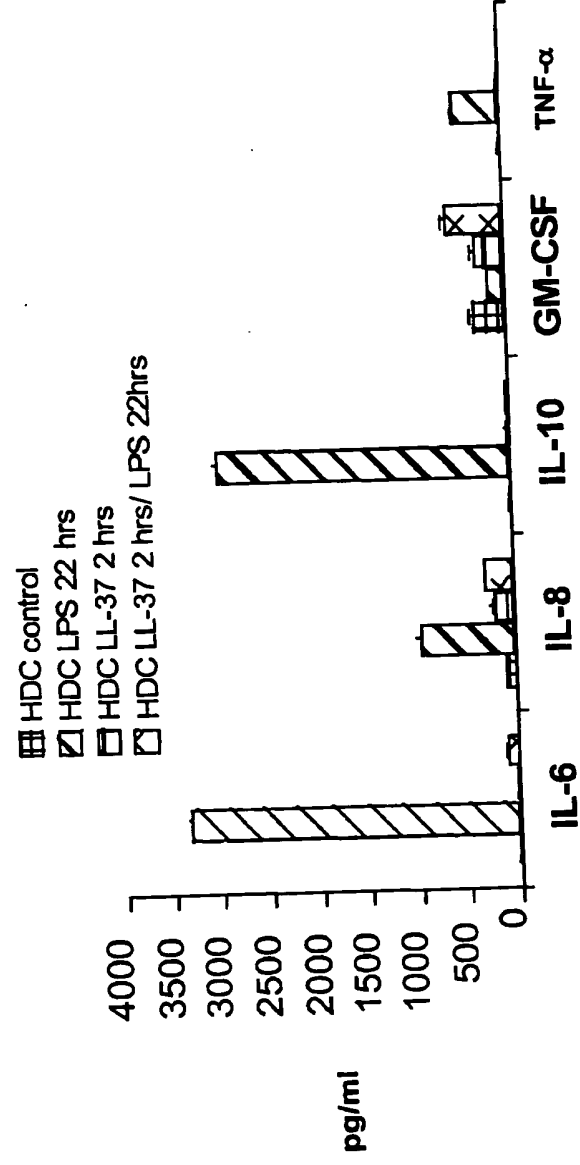
### **Caths**

- Induce proteoglycans  
Gallo et al. (1994) PNAS
- Bind p47 Phox in NADPH oxidase  
Shi. et al. (1996) PNAS
- Bind FPRL1  
De Jang et al. (2000) J Exp Med.
- Inhibits proteasome  
Gao et al. (2000) J. Clin. Invest
- Angiogenic  
Li et al. (2000) Nature Medicine

### **Defensins**

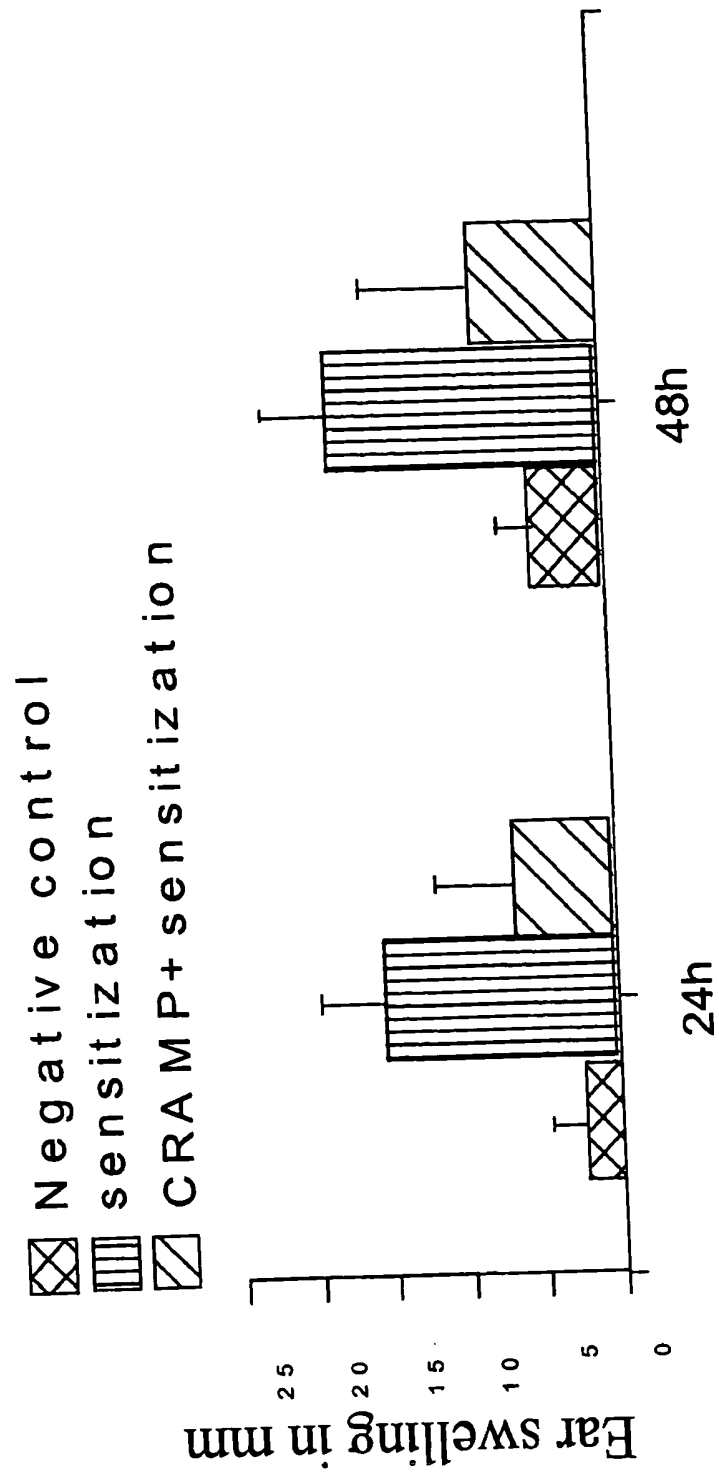
- Binds CCR6  
Yang et al (1999) Science
- Activates TLR-4  
Biragyn et al. (2002) Science

# LL37 blocks LPS-induced chemokine release from Human Dendritic cells

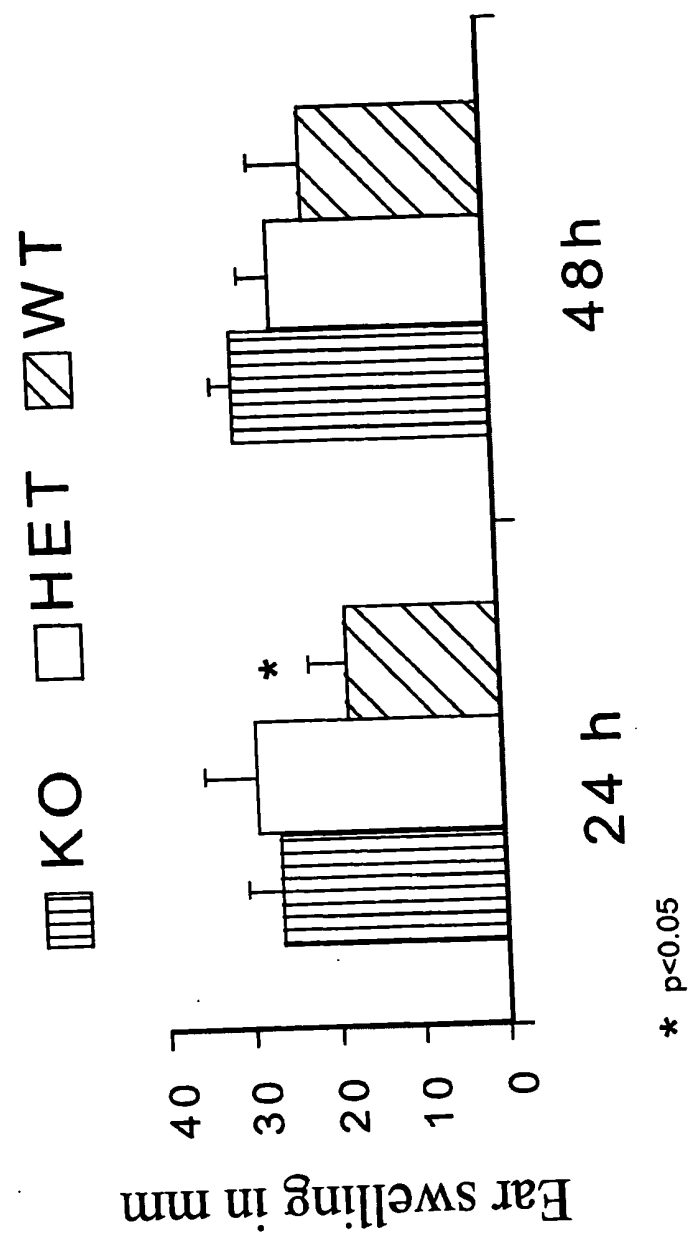




# CRAMP inhibits antigen presentation in vivo



# CRAMP inhibits antigen presentation in vivo



Cathelicidins complex functions as a barrier in the skin

## **FUNCTIONS**

**Antimicrobial**

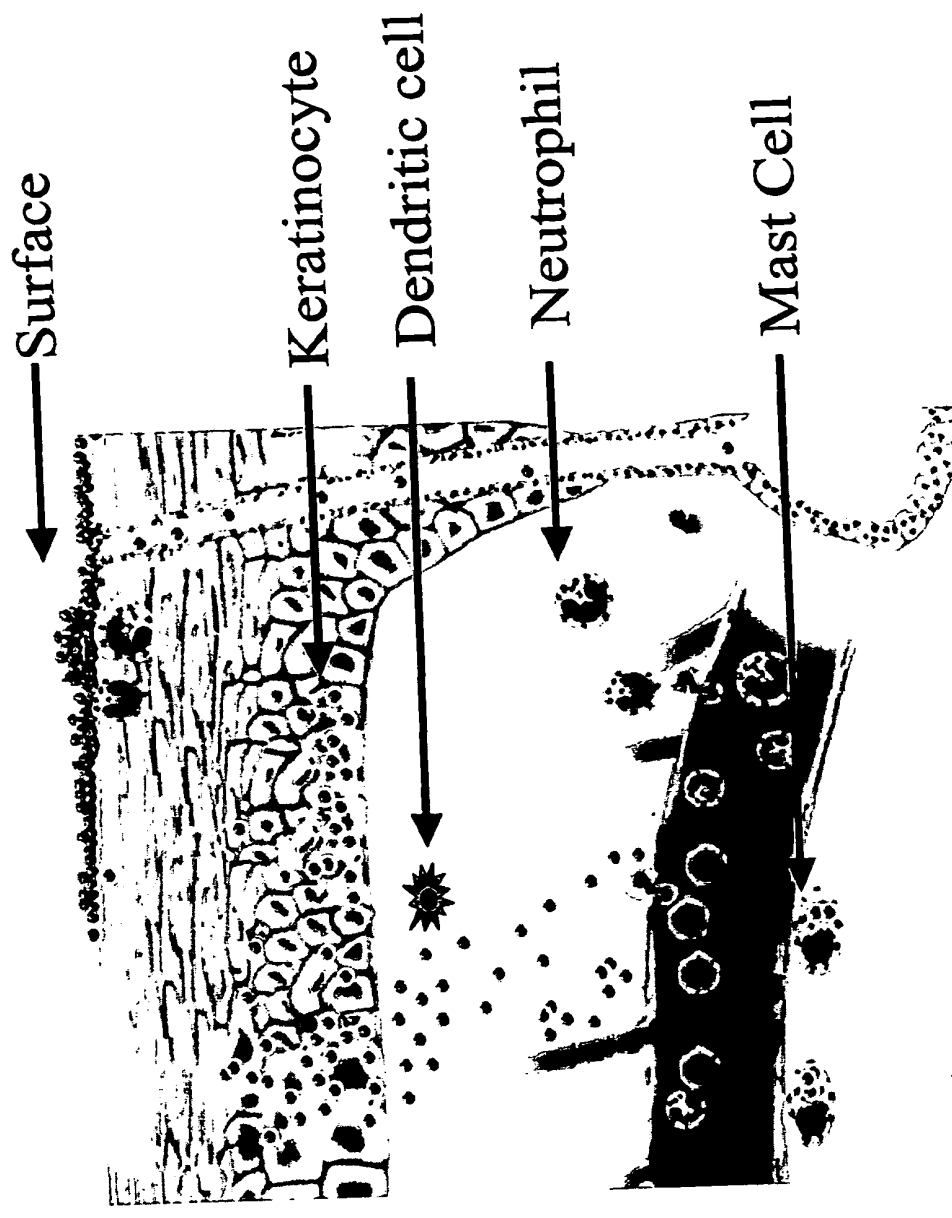
**APC  
maturation**

**cytokine release**

**ECM synthesis**

**Chemotaxis**

**Angiogenic**



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(Form TT-100, 1 July 1997, Modified by UCSD TechTIPS, 16 July 2002)

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**Title of Invention:** The Chip-DasI Technology For Functional Genomics Studies

**SD Case # :** SD2003-244

**Name of Inventor:** Dr. Xiang-Dong Fu

**Mail Code:** MC: 0651

**Department:** Cellular Molecular Medicine

**Campus:** SD

**Home Academic Unit:** \_\_\_\_\_ (please complete)

**Company Name:** AVIVA Antibody Corporation

**Address:** 6160 Lusk Blvd., Suite C204  
San Diego, CA 92121

### **TO BE COMPLETED BY INVENTOR**

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